Dermatotoxicology

Eighth Edition

Edited by
Klaus-Peter Wilhelm
Hongbo Zhai
Howard I Maibach

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Klaus-Peter Wilhelm MD

Department of Dermatology, Medical University of Lübeck and proDERM Institut fur Angewandte Dermatologische Forschung GmbH, Schenefeld/Hamburg, Germany

Hongbo Zhai MD

Department of Dermatology, University of California School of Medicine, San Francisco, California, USA

Howard I. Maibach MD

Department of Dermatology, University of California School of Medicine, San Francisco, California, USA

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Published by Informa Healthcare, 119 Farringdon Road, London EC1R 3DA, UK.

Simultaneously published in the USA by Informa Healthcare, 52 Vanderbilt Avenue, 7th Floor, New York, NY 10017, USA.
Informa Healthcare is a trading division of Informa UK Ltd, Registered Office: Informa House, 30–32 Mortimer Street, London W1W 7RE, UK. Registered in England and Wales number 1072954.

A CIP record for this book is available from the British Library
Library of Congress Cataloging-in-Publication Data available on application

ISBN: 978-1-84184-855-6
eISBN: 978-1-84184-857-0

Orders may be sent to: Informa Healthcare, Sheepen Place, Colchester, Essex CO3 3LP, UK
Telephone: +44 (0)20 7017 6682
Email: Books@Informa.com
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Typeset by Exeter Premedia Services, Chennai, India
Printed and bound in the UK

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Contributors

Heinz William Ahlers
Technology Evaluation, National Institute for Occupational Safety and Health, Pittsburgh, Pennsylvania, USA

Iris S. Ale
Department of Dermatology, University Hospital, Republic University, Montevideo, Uruguay

Divya K. Alla
Private practice, Los Angeles, California, USA

Angela N. Anigbogu
Endo Pharmaceuticals Inc., Westbury, New York, USA

Joao Barroso
Colipa, Brussels, Belgium

David A. Basketter
DABMEB Consultancy Ltd, Sharnbrook, UK

Enzo Berardesca
San Gallicano Dermatological Institute, Rome, Italy

Ludivine Bernard
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Giovanni Brambilla
Department of Internal Medicine, University of Genoa, Genoa, Italy

Andreas J. Brandstetter
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Robert L. Bronaugh
Consultant, Office of Colors and Cosmetics, Food and Drug Administration, College Park, Maryland, USA

Derk P. Bruynzeel
Department of Dermatology, Free University Hospital, Amsterdam, The Netherlands

Javier Camacho
Department of Pharmacology, Centro de Investigación y de Estudios Avanzados del I.P.N., México D.F., Mexico

Stephanie Chao
Stanford Asian Liver Center, Stanford University Medical Center, Stanford, California, USA

Audris Chiang
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Robert P. Chilcott
Chemical Hazards and Poisons Division, Health Protection Agency, Didcot, UK

Myeong Jun Choi
Korea Clinical Research Center Co. Ltd, Gyeonggi-do, Korea

Thomas W. Clarkson
Department of Environmental Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York, USA

Rebecca J. Dearman
Faculty of Life Sciences, University of Manchester, Manchester, UK

Frank Dreher
Neocutis Inc., San Francisco, California, USA

Dena Elkeeb
Division of Hospital Medicine, General and Community Pediatrics, Cincinnati Children's Hospital, Cincinnati, Ohio, USA

Chantra Eskes
SeCAM, Agno, Switzerland

Miranda A. Farage
Feminine Care Innovation Center, Procter and Gamble, Cincinnati, Ohio, USA

Rolf Fautz
Kao Europe Research Laboratories,
Kao Germany GmbH, Darmstadt, Germany

Anna Flammiger
Department of Oncology and Hematology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Anne Fuchs
Kao Europe Research Laboratories,
Kao Germany GmbH, Darmstadt, Germany

G Frank Gerberick
Miami Valley Innovation Center, Procter and Gamble, Cincinnati, Ohio, USA

Josephine Gerby
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Ana M. Gimenez-Arnau
Department of Dermatology, Hospital del Mar, Barcelona, Spain

Elena Gimenez-Arnau
Laboratoire de Dermatochimie, Institut le Bel, Université de Strasbourg (CNRS-UMR 7177), Strasbourg, France

C. L. Goh
National University of Singapore and National Skin Centre, Singapore

Margarida Gonçalo
Clinic of Dermatology, Faculty of Medicine, University of Coimbra, and Coimbra University Hospital, Coimbra, Portugal

Alan H. Hall
Toxicology Consulting and Medical Translating Services, Inc., Laramie, Wyoming, and Colorado School of Public Health, University of Colorado-Denver, Denver, Colorado, USA

Thomas Hartung
Center for Alternatives to Animal Testing (CAAT), Department of Environmental Health Sciences, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA

Kathryn L. Hatch
Agricultural and Biosystems Engineering, The University of Arizona, Tucson, Arizona, USA

Philip G. Hewitt
Toxicology, Merck Serono, Darmstadt, Germany

Anaheed Heydari
Department of Psychology, University of California, Los Angeles, California, USA

Panthea Heydari
Department of Neuropsychiatry, University of California, Los Angeles, California, USA

Sebastian Hoffmann
seh consulting + services, Cologne, Germany

Jurij J. Hostynek
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Xiaoying Hui
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Tanzina Islam
Saskatoon, Saskatchewan, Canada

Jeanette Kaae
Department of Dermato-Allergology, National Allergy Research Centre, Copenhagen and University Hospital Gentofte, Denmark

Nahoko Kaniwa
Division of Medicinal Safety Science, National Institute of Health Sciences, Tokyo, Japan

Molly L. Kile
College of Public Health and Human Sciences, Oregon State University, Corvallis, Oregon, USA

Jong-Heon Kim
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Ian Kimber
Faculty of Life Sciences, University of Manchester, Manchester, UK

Sailesh Konda
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Antti I. Lauerma
Control of Hypersensitivity Diseases, Finnish Institute of Occupational Health, Helsinki, Finland

Cheon Heol Lee
Department of Dermatology, Kangnam Sacred Heart Hospital, Hallym University College of Medicine, Seoul, Korea

Ernest Lee
Dermatology, South California Permanente Medical Group, South Vermont Avenue, Lakeside Building, Harbor City, California, USA

Ivy Lee-Keltner
Stanford University Medical Center, Stanford, California, USA

Cheryl L. Levin
Department of Dermatology, Harvard Vanguard Medical Associates and Harvard Medical School, Boston, Massachusetts, USA

Yakir S. Levin
Department of Dermatology, Boston University, Boston Medical Center, Boston, Massachusetts, USA

Maureen Lloyd
Stony Brook University School of Medicine, Stony Brook, New York, USA

Monika Lindemann
Institute for Transfusion Medicine, University Hospital Essen, Essen, Germany

Howard I. Maibach
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Inge Mangelsdorf
Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany

Slaheddine Marrakchi
Department of Dermatology, Hedi Chaker University Hospital, Sfax, Tunisia

Antonietta Martelli
Unit of Pharmacology, Department of Internal Medicine, University of Genoa, Genoa, Italy

Melissa Martin
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Nikolay V. Matveev
Medical Center for New Information Technologies, Research Institute for Pediatrics and Children's Surgery, and Department of Medical Cybernetics and Informatics, Russian National Research Medical University, Moscow, Russia

James N. McDougal
Department of Pharmacology & Toxicology, Boonshoft School of Medicine, Wright State University, Dayton, Ohio, USA

Kazuhiro Mio
Life Science Research Center, Lion Corporation, Kanagawa, Japan

Bobeck S. Modjtahedi
Eye Center, University of California Davis, Sacramento, California, USA

Richard P. Moody
Healthy Environments and Consumer Safety Branch, Environmental Health Centre, Health Canada, Ottawa, Ontario, Canada

Nathalie L. Moulton-Levy
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Nader Movassagh
Tehran Azad University, Tehran, Iran

Kaley A. Myer
University of California San Francisco, San Francisco, California, USA

Jesper Bo Nielsen
Risk Analysis and Risk Communication, Institute of Public Health, University of Southern Denmark, Odense, Denmark

Barbara Noury
Department of Dermatology, University of California San Francisco, San Francisco, California, USA

Ning Pan
Department of Biological System Engineering, University of California, Davis, California, USA

Alessandra Pelosi
San Gallicano Dermatological Institute, Rome, Italy

Iván Restrepo-Angulo
Department of Pharmacology, Centro de Investigación y de Estudios Avanzados del I.P.N., México D.F., Mexico

James V. Rogers
Battelle, Columbus, Ohio, USA

Cindy A. Ryan
Miami Valley Innovation Center, Procter and Gamble, Cincinnati, Ohio, USA

Yoshiro Saito
Third Section, Division of Drugs, National Institute of Health Sciences, Tokyo, Japan

Annahita Sarcon
UC Davis School of Medicine, Sacramento, California, USA

Pietro Sartorelli
Unit of Occupational Medicine and Toxicology, University of Siena, Siena, Italy

Tsippora Shainhouse
Dermatology, South California Permanente Medical Group, South Vermont Avenue, Lakeside Building, Harbor City, California, USA

Raja K. Sivamani
UC Davis Department of Dermatology, Sacramento, California, USA

Sahar Sohrabian
Dermatology, Olive View UCLA Medical Center, Sylmar, California, USA

William S. Stokes
National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, Division of the National Toxicology Program, National Institute of Environmental Health Sciences, National Institutes of Health, U.S. Department of Health and Human Services, Research Triangle Park, North Carolina, USA

Judy Strickland
Integrated Laboratory Systems, Inc., Research Triangle Park, North Carolina, USA

Haw-Yueh Thong
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Jacob P. Thyssen
Department of Dermato-Allergology, National Allergy Research Centre, Copenhagen, and University Hospital Gentofte, Denmark

Miriam Cortés Torres
Department of Toxicology, Centro de Investigación y de Estudios Avanzados del I.P.N., México D.F., Mexico

Jackie M. Tripp
Tripp Dermatology, Delray Beach, Florida, USA

Germaine L. Truisi
Toxicology, Merck Serono, Darmstadt, Germany

Tsen-Fang Tsai
Department of Dermatology, National Taiwan University Hospital, and National Taiwan University College of Medicine, Taipei, Taiwan

Peter Ulrich
Safety Profiling and Assessment, Novartis Pharma AG, Basel, Switzerland

Niels K. Veien
The Dermatology Clinic, Aalborg, Denmark

Andrea De Vizcaya-Ruiz
Department of Toxicology,
Centro de Investigación y de Estudios Avanzados del I.P.N., México D.F., Mexico

Hans-Werner Vohr
Toxicology, Bayer Healthcare AG, Wuppertal, Germany

Jens-Uwe Voss
Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany

Aziza A. Wahby
Touro College of Osteopathic Medicine, New York, New York, USA

Christina Y. Wang
Occupational and Environmental Medicine, University of California, San Francisco and Kaiser Occupational and Environmental Medicine, San Francisco, California, USA

Naissan I. Wesley
Private practice, Los Angeles, California, USA

Ronald C. Wester
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Klaus-Peter Wilhelm
proDERM Institute for Applied Dermatological Research, Schenefeld/Hamburg, Germany
Department of Dermatology, Medical University of Lübeck, Lübeck, Germany

Katherine Willard
Stanford University Medical Center, Stanford, California, USA

Malcolm M.Q. Xing
Department of Mechanical Engineering, University of Manitoba, Winnipeg, Manitoba, Canada

Danny Zaghi
Department of Dermatology, University of California San Francisco, San Francisco, California, USA

Grazyna Zareba
Department of Environmental Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York, USA

Hongbo Zhai
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Yue Zheng
Department of Dermatology, University of California Medical School, San Francisco, California, USA

Wen Zhong
Department of Textile Sciences and Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada

Kristine B. Zitelli
Department of Dermatology, University of Cincinnati, Cincinnati, Ohio, USA

1 Pharmacogenetics and dermatology

Tsippora Shainhouse, Ernest Lee, and Howard I. Maibach

INTRODUCTION

Pharmacogenetics, Adverse Drug Reactions, and Personalized Medicine

Pharmacogenetics is the study of the role that inheritance plays in the individual variation in drug response. The response spectrum of a drug may range from life-threatening adverse drug reactions (ADRs) to inadequate therapeutic effects. For the clinician, this concept is relevant when asking why a drug is expectedly efficacious in one segment of the population, ineffective for another, and toxic or even fatal for a third. Identification of genetic variations that result in differences in drug bioavailability, biotransformation and, ultimately, clinical response is the key to the new era of "personalized medicine." Personalized medicine promises to deliver safer, more effective therapies to patients by down-playing the one-drug-fits-all theory, in exchange for recognizing the impact of a person's specific genetic make-up on the pharmacodynamics (PD) and pharmacokinetics (PK) of a specific drug, and integrating this information to develop a personalized therapeutic plan (1).

PK describes what the body does to a drug to make it available for use. A drug's PK properties are determined by genes that direct the disposition [absorption, distribution, metabolism, excretion (ADME)] of a drug in the body (2). Drug-metabolizing enzymes, specifically those of the cytochrome p450 family, and drug transporter proteins, such as P-glycoprotein (P-gp) transporters, play a key role in this process. These particular enzymes are governed by allelic variations within both similar and ethnically diverse populations. The most common functional consequence of these variations is concentration-related toxicity, either due to the accumulation of prodrug (consider an azathioprine patient with nonfunctional ThiopurineS-methyltransferase (TMPT) alleles will have debilitating myelosuppression) or increased, adverse clinical effect in rapid metabolizers (ultra-rapid codeine-converting mothers with a specific CYP2D6*2 $\times$ 2 allele can inadvertently kill their breastfed infants by overdosing them with the morphine endproduct).

PD describes what a drug does to the body, that is, the clinical impact. For example, variation in the intrinsic amount of VKORC1 gene product (vitamin K epoxide reductase) that an individual has will impact the effect that warfarin has on bleeding tendency. Certain alleles/haplotypes are more common in specific ethnic populations. Haplotype-specific guidelines have been published to determine the ideal starting dose to attain and maintain a therapeutic International Normalized Ratio (INR).

In children, we must consider not only differences in genotype, but to some degree, variation in gene expression during growth and development (3). Although TMPT enzyme activity is most likely present at birth, and CYP2D6 and CYP3A4 are acquired in the first few weeks of life, delayed maturation of other drug-metabolizing enzymes can contribute to concentration-dependent toxicities, and altered concentrations of circulating plasma proteins can affect drug distribution (cephalosporins in neonates).

More than 2 million cases of ADRs, including 100,000 deaths, are reported annually in the United States (4,5). They account for 2.4–12% of hospital admissions, 4.6% of deaths in hospitalized patients, and have been reported to be the 4th leading cause of death in hospitalized patients (6). This costs the US over $177 billion annually (7).

In situations in which genetic risk factors can accurately predict risks for serious ADRs, either idiosyncratic or dose-related, drug-specific pharmacogenomic biomarkers are invaluable in the prevention of these ADRs and in tailoring clinical treatment decisions.

However, to be successful clinical tools, biomarkers should have high positive and negative predictive values, be simple to perform and interpret, be easy to repeat, sourced from easily accessible body fluids or tissue, and be cost-effective (8).

The US Food and Drug Administration (US FDA) has already approved labels on various drugs to include information associated with human genomic biomarkers. In specific cases, recommendations are made for pharmacogenetic testing before initiating treatment (warfarin, thiopurines, carbamazepine in Asian patients, abacavir), and in others, dose selections are offered (7,9).

ANTIMETABOLITES

Azathioprine and 6-Mercaptopurine

Thiopurine drugs, including 6-mercaptopurine (6-MP) and azathioprine, a prodrug that is converted to 6-MP in vivo, are cytotoxic and immunosuppressant medications used in the management of autoimmune connective tissue disease, immune-bullous skin disease, atopic dermatitis, neutrophilic dermatoses, photodermatoses, and as an antirejection drug in organ transplant patients. These drugs have a narrow therapeutic window with the potential for life-threatening myelosuppression (10).

Once azathioprine is absorbed and converted to 6-MP in the red blood cell (RBC), it can undergo one of three competing processes. Therapeutically, it is intended to be anabolized by the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) to its active form, the purine nucleotide analog, 6-thioguanine (6-TGN). 6-TGN can then be incorporated into DNA strands, thus suppressing DNA replication and new cell formation. The other two pathways halt this process and create inactive metabolites by one of two processes: oxidation by xanthine oxidase (which will be discussed further in the following section); or methylation by

TABLE 1.1
Frequency of Thiopurine S-Methyltransferase Alleles by Race (%) (9)

Allele	Caucasian	African American	Asian
TPMT*2	0.2	0.4	0
TPMT*3A	3–5	0.4–0.8	0
TPMT*3C	0.2	2–7	2–5

thiopurine S-methyltransferase (TPMT), a cytosolic drug-metabolizing enzyme. In fact, the level of measured TPMT in RBCs is inversely proportional to the concentration of 6-TGN in RBCs. Genetic polymorphisms of TPMT are associated with TPMT activity. Thus, patients with a genetic predisposition for high enzyme activity may be chronically underdosed, whereas patients with low TPMT activity are prone to developing toxic levels of 6-TGN and are at high risk for severe leukopenia, and even death from standard dosing. An 89% of the population has clinically normal TPMT activity, associated with inheritance of at least one wild-type allele, TPMT*1. An 11% of the population has intermediate levels of TPMT activity, and 1/300 of people inherit low or absent TPMT activity, as an autosomal recessive trait. Among the low-activity population, three alleles account for 95% of these inherited cases: TPMT*3A, the most common variant in Caucasians, TPMT*3C, the most common variant in East Asians and African Americans, and TPMT*2. TPMT*3A and *3C alleles result in virtually no enzyme activity, whereas *3B and *2 yield significantly decreased enzyme activity (Table 1.1) (9,11).

Patients who are homozygous for alleles that result in low or no enzyme activity must be treated with 1/10–1/15 the standard doses of 6-MP and azathioprine, and they must be monitored carefully with serial complete blood counts throughout the treatment (11). TPMT phenotyping is more common than genotyping, and considered to be more reliable in predicting and averting thiopurine toxicity and myelosuppression (12). Six separate economic evaluations of TPMT testing for patients prescribed thiopurine drugs recommended that TPMT is a cost-effective preventative measure (13). As such, TPMT enzyme testing must be determined before initiating treatment, to avoid both underdosing and toxicity.

Allopurinol

Decreased activity of xanthine oxidase is not related to genetic variability but rather to drug interactions. Allopurinol inhibits xanthine oxidase in the 6-MP metabolic process, thus shunting more substrate through the HGPRT pathway, yielding increased, immunosuppressive levels of 6-TGN that can lead to significant myelosuppression. If a patient requires both allopurinol and azathioprine, the azathioprine dose must be reduced by 75% (14).

Interestingly, allopurinol is the most common cause of toxic epidermal necrolysis (TEN) in Europe and Israel (15). Carriers of the HLA-B*5801 allele have an increased risk of severe cutaneous adverse reactions to allopurinol, including hypersensitivity reactions, Stevens–Johnson syndrome (SJS) and TEN (16). This is most notable in Han Chinese patients in Taiwan, as well as Japanese and Thai patients (17), and it is suggested that this biomarker be tested before initiating treatment in Asian patients, in particular (18).

Methotrexate

Methotrexate is an anti-inflammatory and immunosuppressive drug that is commonly used in the management of psoriasis, as well as other immunobullous and autoimmune connective tissue dermatoses. It acts as a competitive antagonist of the enzyme dihydrofolate reductase, thus preventing the conversion of dihydrofolate to tetrahydrofolate, a co-factor in the production of purine nucleotides for DNA and RNA synthesis. By inhibiting DNA synthesis in competent lymphocytes, it acts as an immunosuppressive agent.

Allelic variations in the gene for 5,10-methylene-tetrahydrofolate reductase (MTHFR) enzyme, specifically at the 677 codon, can be used to predict lymphocyte sensitivity to methotrexate. Studies have demonstrated that lymphocytes heterozygous for the mutant allele MTHFR 667T are significantly more sensitive to methotrexate than those carrying the homozygous wild-type allele MTHFR 667C, suggesting that this pharmacogenetic biomarker may be considered in the calculation of methotrexate dosing (19). The impact of a second MTHFR polymorphism at codon 1298 (C is more sensitive than A) is not as strong as the 667 locus; however, the combined heterozygous state (677CT/1298AC) in patients who do not receive folate supplementation together with their methotrexate, yields a lower rate of hepatotoxicity (20).

A recent review of eight different polymorphisms in five of the enzymes involved in folate, purine, and pyrimidine metabolism in psoriatic patients being treated with methotrexate revealed some relevant biomarkers, with an even more clinically relevant intervention strategy (20). Patients with the reduce folate carrier (RFC) 80A allele (wild type is G) have no therapeutic response to methotrexate, and have such a high incidence of adverse side effects, and tend to self-select by discontinuing treatment. Similarly, patients with the 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (ATIC) 347G polymorphism in the ATIC gene have more severe side effects that leave patient self-selecting to discontinue therapy. Polymorphisms in the thymidylate synthase (TS) 5-UTR gene not only demonstrate poor therapeutic response, but significant adverse drug events. Psoriatic patients with the TS 5-UTR 3R allele have a very poor therapeutic response to methotrexate, if they have palmoplanatar psoriasis, but all patients with this allele receiving methotrexate without folic acid supplementation have a 12–15× increase in ADRs, including a 13× incidence of hepatotoxicity. Similarly, psoriatics with the TS 5-UTR 6bp del allele have an 8× increased risk for a significantly elevated alanine transaminase with unsupplemented methotrexate treatment. As such, the impact of many polymorphism-related ADRs in psoriatic patients on methotrexate therapy can be reduced or eliminated with folic acid supplementation.

5-Fluorouracil

TS catalyzes the conversion of deoxyuridylate and 5,10-methylenetetrahydrofolate (CH_2H_4 folate) to deoxythymidine monophosphate (dTMP) and 7,8-dihydrofolate. This reaction is the sole de novo biosynthesis of thymine in DNA, and therefore inhibition of TS blocks DNA synthesis, thereby causing cell death.

5-Fluorouracil (5-FU) is a fluorinated pyrimidine analog (the prodrug of 5-fluoro-2-deoxyuridine monophosphate (FdUMP) that covalently binds to TS, thus inactivating the anabolic enzyme complex and preventing the conversion of deoxyuridine monophosphate

to dTMP, which is required for DNA synthesis. 5-FU also incorporates itself into RNA strands as an abnormal base pair, thus inhibiting cell growth. Clinical data have suggested that response to 5-FU-based chemotherapy regimens is inversely associated with intratumoral TS mRNA and protein expression (21). There are three functional gene polymorphisms that regulate TS expression, help prognosticate disease-free and overall survival, as well as predict therapeutic benefit of 5-FU (1).

5-FU is currently one of the most widely administered chemotherapeutic agents used for the treatment of epithelial cancers. Systemic 5-FU (intravenous administration) is poorly absorbed; 20% is anabolized to the active metabolite, whereas 80% is quickly catabolized by the liver and excreted in the urine. Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme in the catabolism and clearance of 5-FU. Expression of DPD has been related to tolerance and response to 5-FU-based therapy. Low expression or absence of DPD is associated with 5-FU accumulation and increased risk of severe toxicity; high expression of DPD is associated with poor response to 5-FU therapy. Molecular studies have suggested that there is a relationship between allelic variants in the DPYD gene (the gene that encodes DPD), found on chromosome 1p22, and a deficiency in DPD activity, thus providing a potential pharmacogenetic basis for 5-FU toxicity. A 3–5% of the population has low or no DPD activity. Four allelic variants have been demonstrated to have clinical relevance. The c1905 + 1 G > A (otherwise known as IVS14 + 1G > A or DPD*2A) is a splice variant leading to zero enzyme activity. However, this phenotype is not clinically apparent in the presence of a second wild-type allele. The c1679 T > G mutation (isoleucine to serine at codon 560) and c2846 A > T (aspartic acid to valine at codon 949) produce low enzymatic activity. A deep intronic (noncoding) slicing mutation (c1129–5923 C > G) is relevant in the European population (22).

Some studies have correlated tumoral DPD activity with 5-FU response, suggesting it may be a useful pharmacogenomic marker of patient response to 5-FU-based chemotherapy (23). It is possible that tumor DPD activity may predict the clinical severity of a patient's response to topical 5-FU application in the treatment of nonmelanoma skin cancers (NMSCs), including erythema, swelling, and treatment efficacy.

ANTICONVULSANTS

Carbamazepine

Human leukocyte antigen (HLA) allelic biomarkers can be helpful in predicting ADRs, particularly in patients at risk for severe hypersensitivity reactions. Symptoms of these systemic events include rash (often macular–papular exanthems), fever, malaise, nausea, headache, and myalgias, and usually develop within 6 weeks of starting a given medication. Discontinuation of the medication leads to symptom resolution, but re-introduction of the same medication can produce an immediate-type hypersensitivity reaction that results in severe hypotension, respiratory failure, and even death.

Carbamazepine, a firstline agent in seizure management, and now used off-label in the treatment of headache, chronic pain, trigeminal neuralgia and mood disorders can cause SJS and TEN. Other anticonvulsants are associated with similar ADRs. In 2007, the US FDA added a blackbox warning to the drug's label, recommending that Asian patients be tested for the HLA-B*1502 allele, a demonstrated biomarker for carbamazepine-associated SJS–TEN, before initiating therapy (9,24). Asians and patients with Asian ancestry have a 98% incidence of carbamazepine-induced SJS–TEN if they carry the HLA-B*1502 allele. The frequency of this allele is highest in South Asians (Han and Hong Kong Chinese, Taiwanese, Thai, Indians; 8–11%) *versus* North Asians (Beijing Chinese, Japanese, Koreans; 1–2%) (25). Interestingly, other races carrying this allele do not have the increased risk of developing SJS–TEN (26,27).

A new allelic variant HLA-A*3101 has been determined to be a biomarker for carbamazepine-induced hypersensitivity in Caucasians of European descent. The skin findings may range from maculopapular exanthems to severe blistering reactions. With a 5–10% prevalence of carbamazepine-associated hypersensitivity in Europeans, investigators have suggested that recommendation to screen for this second, important biomarker be added to the drug's US FDA labeling (28).

Dilantin + Fluconazole/Rifampin

Dilantin (valproic acid) is considered to be one of the safer antiepileptic drugs, less likely to induce a hypersensitivity-type reaction at standard doses. Metabolized by the p450 enzyme, CYP2C9, serum concentration of valproic acid will be affected by drug–drug interactions with common dermatology drugs, which may inhibit (e.g., fluconazole) or induce (e.g., rifampin) the CYP2C9 enzyme.

ANTIRETROVIRALS

Human immunodeficiency virus (HIV) is an increasingly difficult virus to treat because it is continually mutating. Most patients require a cocktail of medications to attempt to halt viral replication at various steps in its life cycle. Interestingly, patients who are homozygous for null alleles in the chemokine receptor (CCR)-5 gene are resistant to HIV infection and do not contract the disease (29). Other patients carry gene polymorphisms that predispose them to severe adverse reactions to specific antiretroviral drugs.

Abacavir

Abacavir is a nucleoside analog inhibitor of HIV-1 reverse transcriptase that is used in combination with other antiretrovirals (usually lamivudine and ritonavir) as an effective means of retarding susceptible HIV strains. Approximately 4.3% of patients (Caucasian > African American) have developed a severe drug hypersensitivity reaction to this medication, presenting with fever, rash, malaise, headache, acute respiratory symptoms, and even life-threatening hypotension and cardiovascular collapse, if the medication is not discontinued. It typically appears within the first six weeks of initiating treatment (median time of onset is 11 days) (30). The HLA B*5701 allele was identified as a risk factor for abacavir hypersensitivity. Because a second exposure to the drug yields an immediate-type hypersensitivity reaction, which can lead to angioedema and death, cutaneous patch testing was used to corroborate and increase the specificity of the clinical diagnosis (31,32). Recent studies have since demonstrated the cost-effectiveness of HLA B*5701 genotyping to screening patients prior to initiating abacavir therapy (33,34). HLA-DR7 and HLA-DQ3 have also been associated with abacavir hypersensitivity.

Neveripine

Cutaneous reactions are common in patients being treated with non–nucleoside reverse transcriptase inhibitors (NNRTIs). Rashes develop in about 15% of patients on neviripine, 1.5% of which are severe; it is associated with a 0.301% incidence of SJS (1). The rash is usually noted within the first 2–4 weeks of initiating treatment, and is not expected to develop beyond the first three months. Drug-induced hypersensitivity syndrome (DRESS) has been associated with the HLA-DRB*0101 polymorphism. It occurs more commonly in women and in patients with higher CD4 counts at the initiation of therapy (>250 cells/mm^3 in women or >400 cells/mm^3 in men). Hepatotoxicity is an even more common adverse effect of neviripine therapy. The incidence of neviripine-induced hepatotoxicity may actually be decreased in patients with the MDR1 *3434T allele (1). Neviripine is metabolized in the liver by CYP3A4 and CYP2D6 enzymes. Neviripine levels may be increased or decreased in patients taking concomitant medications that utilize these same enzymes. This in turn would lead to changes in efficacy as well as the frequency and intensity of adverse side effects.

Efavirenz

Efavirenz, one of the most potent NNRTIs (14), is metabolized in the liver by CYP3A4 and CYP2B6 enzymes. It is well tolerated, but is often associated with rashes. Forty percent of pediatric HIV patients have presented with rash during treatment, but only 1% have developed SJS (1). Unlike neviripine treatment, it is not necessary to discontinue this drug for mild-to-moderate rashes, as they tend to resolve with time. However, concomitant medications that induce CYP3A4 and CYP2B6 enzymes can reduce the levels of efavirenz, thus reducing its efficacy (14).

ANTIFUNGALS

Warfarin interaction

The azole drugs interfere with CYP 2C9 (fluconazole is a potent inhibitor) and CYP 3A4 (ketoconazole and itraconazole are potent inhibitors). Any drug using these pathways may have its metabolism altered when given concomitantly with an azole antifungal agent (35). Excessive anticoagulation can occur with a significant increase in INR values when fluconazole, (36) ketoconazole, (37), or itraconazole (38) are taken with warfarin.

Simvastatin myopathy

Rhabdomyolysis is described as an adverse event of simvastatin therapy either by itself or in combination with other medications. The antifungal ketoconazole increases the possibility of rhabdomyolysis developing from the use of simvastatin (39). Ketoconazole is an antifungal sterol synthetic inhibitor of the azole group. Azole antifungals inhibit the CYP3A4-mediated metabolism of simvastatin resulting in increased serum levels and effects of simvastatin.

Co-administration of simvastatin with itraconazole in healthy volunteers has led to rises of over 10-fold in the area under the curve) and C_{max} (maximum concentration) of simvastatin (40). Case reports also document rhabdomyolysis with concurrent use of fluconazole (41). Lowest possible doses of statins should be used if co-administration of the azoles cannot be avoided. Patients should be advised to report any unexplained muscle pain, tenderness, or weakness.

Voriconazole

Voriconazole is a broad spectrum triazole antifungal agent available as both an oral and intravenous formulation. It has potent in vitro as well as in vivo activity against a broad spectrum of pathogens, including *Aspergillus*, *Candida*, and *Cryotococcus* (42). Voriconazole metabolism is highly affected by the CYP2C19 enzymes. CYP2C19 is the least expressed CYP2C isozyme in the liver. Despite this, its polymorphisms can affect the metabolism of several classes of drugs, including antipsychotics, antidepressants, and proton pump inhibitors (43). Carriers of two null alleles display the poor metabolizer (PM) phenotype, whereas extensive metabolizers (EMs) carry at least one functional allele. Heterozygous EMs are sometimes referred to as intermediate metabolizers (IMs) (44). Approximately 20% of Asians but only 3–5% of Caucasians and Africans are PMs. The two most common defective alleles are CYP2C19*2 and CYP2C19*3, the latter occurring primarily in Asians. By contrast the CYP2C19*4 allele is more common in Caucasian (frequency = 0.6%) and accounts for at least 5% of the PMs in Caucasians (45). Most recently, CYP2C19*17 was detected; it is associated with increased CYP2C19 activity due to increased gene transcription. It is rare in Asians but quite common in Africans and Europeans (46). Because voriconazole is primarily metabolized by the CYP2C19 isozyme, genotyping may have clinical utility, particularly because voriconazole has a somewhat narrow therapeutic index (47).

ANTIBIOTICS

Antibiotic use in dermatology can be affected by the genetic polymorphisms that alter metabolism of these medications. Some of the pertinent pathways for metabolism of dermatologic drugs include (*i*) *N*-acetylation and (*ii*) CYP enzymes.

Metabolism by *N*-Acetylation

Individuals who are rapid acetylators excrete the target drugs rapidly, and therefore experience higher than expected rates of treatment failure (48). In addition, rapid acetylators require higher doses of medication for clinical effect. Individuals who are slow acetylators are more likely to develop side effects from medications: these include neuropathy from isoniazid; drug-induced lupus from procainamide and hydralazine; and TEN from sulfonamides (49). Individual differences in metabolism may predispose patients to idiosyncratic reactions from antibiotics metabolized by this pathway.

Sulfonamides

Sulfonamides are metabolized by *N*-acetylation (mediated by a genetically polymorphic enzyme) and oxidation to potentially toxic metabolites. Those who are slow acetylators appear to be most at risk (50). In particular, the slow acetylator phenotype is a risk factor for SJS/TEN. Wolkenstein et al., looked at 32 inpatients admitted for sulfonamide- or anticonvulsant-induced SJS/TEN as well as a control group of 20 healthy volunteers; 17/18 patients with sulfonamide-induced SJS/TEN were slow acetylators compared with 8/14 patients with anticonvulsant-induced SJS/TEN *versus* 10/20 healthy volunteers (51).

Isoniazid

In the 1950s, a high variation in individual rates of excretion of isoniazid was observed among people being treated for tuberculosis (52).

Following a single oral dose of isoniazid, a bimodal pattern of plasma isoniazid levels was demonstrated, leading to the concept of rapid and slow eliminators of this drug. The genetic basis for this variation arose from the observation that monozygotic and dizygotic twins had a high concordance rate for excretion rates. Further investigation revealed that the enzyme responsible for the metabolism of isoniazid was *N*-acetyltransferase (NAT). This enzyme is central in the metabolism of a wide variety of drugs, all of which contain an arylamine or hydrazine group. The genetic basis for variability in the action of this enzyme results from polymorphisms at the *NAT2* gene locus. Fifteen variant alleles for *NAT2* have been identified. Several of the alleles have been associated with the rapid acetylator phenotype (*NAT2*4*, *NAT2*12*, and *NAT2*13*), whereas others have been associated with slow acetylation (*NAT2*5*, *NAT2*6*, *NAT2*7*, and *NAT2*14S*) (53). In particular, there appears to be an association of the slow-acetylator phenotype and drug-induced liver injury (54).

Rifampin

Potential adverse drug interactions between antibiotics and oral contraceptives are of great relevance in dermatologic practice. The enterohepatic circulation of contraceptive steroids can be interfered with by antibiotic effects on bacterial flora in the bowel, and lower serum levels of the contraceptives can result. Some have suggested increasing the estrogen component of the pill to 50 µg or adding other forms of birth control for the duration of antibiotic therapy (55). However in practice, the failure of oral contraceptives with oral antibiotics is low (56). In fact, a recent review of the literature suggests that there is little convincing evidence to show a systematic interaction between antibiotics and oral contraceptives other than rifampin (57). Rifampin, an antibiotic used in treating diseases, such as tuberculosis, is a known CYP3A4 and CYP2C9 inducer in vivo (58,59). It has also been suggested that rifampin is an inducer of CYP1A2, CYP2C8, and CYP2C19 (60–63).

Doxycycline

Doxycycline is a CYP3A4 substrate, and hence its metabolism has the potential to be altered depending on the individual genetic profile. Tetracyclines as a group interfere directly with CYP isoforms, and thus influence the metabolism of medications that utilize this pathway. (Please see Ashourian and Cohen for a comprehensive list of possible drug interactions with the tetracyclines (64).) The most relevant dermatologic interactions include (*i*) increasing the level of methotrexate, (*ii*) increasing risk of pseudotumor cerebri with concomitant isotretinoin use, and (*iii*) interference with bactericidal activity of the penicillins, which depend on bacterial wall synthesis for efficacy.

Sirolimus (Formerly Known As Rapamycin)

Rapamycin and its derivatives are immunosuppressive macrolides that block mammalian target of rapamycin (mTOR) function and yield antiproliferative activity against a variety of malignancies (65). Topical rapamycin has shown efficacy in the treatment of angiofibromas in tuberous sclerosis (66). Regarding sirolimus/rapamycin, results from different studies have demonstrated that there is a significant association between sirolimus concentration/dose ratio and CYP3As polymorphisms (67–69). A lower sirolimus concentration/dose ratio was observed in the CYP3A5*1 carriers (*1/*3 or *1/*1) than in the CYP3A5*3/*3 carriers, suggesting that CYP3A5 nonexpressors require lower sirolimus dose to achieve therapeutic concentrations. There is also an association between the CYP3A4*1B polymorphism and higher sirolimus requirement (70–72).

ANTICOAGULANTS

Warfarin (Coumadin)

Warfarin is a racemic, oral anticoagulant prescribed most commonly for the treatment and prevention of thromboembolic events. While usually seen in patients presenting to dermatology with a history of myocardial infarction, stroke and pulmonary emboli, and deep vein thrombosis, it is sometimes necessary to prescribe it for autoimmune, dermatologic-associated diseases, including antiphospholipid syndrome. However, more relevant is the interaction of concomitant dermatology medications that interact with warfarin-metabolizing enzymes.

S-warfarin, which is 3–5 times more potent than R-warfarin, is primarily metabolized by CYP2C9 (9). Polymorphisms in the gene influence drug metabolism and efficacy. The CYP2C9*2 −430 C > T base pair change encodes an arginine to cysteine amino acid change at codon 144, that results in a 30–40% reduction in enzyme activity for S-warfarin metabolism (IM) (73), compared with patients with the wild-type CYP2C9*1/*1 genotype. A second polymorphism of the same gene is the CYP2C9*3 −1075A > C base pair change. This alteration of isoleucine to leucine at the 359 codon, yields an almost complete loss of function of the enzyme (PM), and negligible S-warfarin metabolism. CYP2C9*2, *3 alleles are seen in 8–12% of Caucasians, 1–3% of African Americans, and in <1% of Asians (4). Clinically, these patients require a significantly lower warfarin dose to maintain therapeutic INR levels and to prevent dangerous bleeding events (74). As CYP2C9 enzymes metabolize 10% of all drugs (14), warfarin metabolism can be affected by co-administration of other medications. Antifungals, fluconazole in particular, is a potent CYP2C9 inhibitor; concomitant therapy can result in a markedly elevated level of warfarin.

A third genetic polymorphism that affects warfarin metabolism involves the VKORC1 (vitamin K 2, 3-epoxide reductase complex, subunit 1) gene, the target enzyme of warfarin. The VKORC1–1639 G > A base pair substitution yields an increased level of warfarin active metabolite. The AA genotype is seen in up to 80% of Chinese patients and 14% of Caucasians (4). It is important to lower the initial dose in these patients. New warfarin dosing tables for achieving optimal INR levels, which incorporate both clinical and pharmacogenetic data, have been developed (75). The WRAPID algorithm demonstrates similar time to achieve first therapeutic response and time to stable anticoagulation, which is independent of CYP2C9 or VKORC1 genotype (76).

Clopidogrel (Plavix)

Antiplatelet therapy is a key in the prevention of atherothrombotic disease processes. Dual therapy with clopidogrel and aspirin is most common. Aspirin is discussed in the following section. Clopidogrel is a prodrug that is converted by CYP2C19 enzymes to an active compound that inhibits adenosine diphosphate (ADP)-induced platelet aggregation. Many patients on this treatment regimen continue to develop recurrent thromboembolic and ischemic

events. Genetic polymorphisms in this CYP enzyme explain some of the variability in clopidogrel efficacy (77). Clopidogrel-treated patients with the loss-of-function CYP2C19*2 allele exhibit reduced platelet inhibition compared with those with the wild-type CYP2C19*1 allele, and experience a higher rate of cardiovascular events (78) (this is important because estimates suggest that up to 25% of whites, 30% of blacks, and 50% of Asians carry the loss-of-function allele, which would render them resistant to clopidogrel (79,80). Even patients with reduced-function CYP2C19*3, *4, or *5 alleles may derive less benefit from clopidogrel than those with the full-function CYP2C19*1 allele. Concomitant administration of clopidogrel and proton pump inhibitors, specifically omeprazole, which is often co-administered to prevent gastrointestinal side effect, and is an inhibitor of CYP2C19, produces a small reduction in the inhibitory effects of clopidogrel on ADP-induced platelet aggregation (81). This interaction does not appear to increase the risk of cardiovascular events.

Acetylsalicylic Acid-induced Urticaria

Aspirin [acetylsalicylic acid (ASA)] is an anti-inflammatory drug that acts by acetylating the enzymes in platelets that synthesize thromboxane A2 (TXA2), and at higher levels, prostaglandin inhibitor 2 (PGI2). TXA2 inhibition prevents platelet aggregation, activation, inflammation, and fever reaction. PGI2 inhibition prevents platelet aggregation induced by endogenous vessel wall enzymes, as well as vasodilation. Most patients presenting to the dermatologist are taking low-dose aspirin for the prevention of stroke and myocardial infarction. These patients tend to have ecchymoses in the skin, and bleed easily and longer with cutaneous surgical procedures. However, ASA has numerous off-label dermatologic uses, including, but not exclusive to, erythema nodosum, postherpetic neuralgia, vitiligo, antiphospholipid antibody syndrome, Degos' disease, necrobiosis lipoidica diabeticorum, erythromelalgia, and mastocytosis (14).

However, aspirin-exacerbated respiratory disease (AERD) and aspirin-induced/intolerant urticaria (AIU) are immune-mediated reactions, associated with mast cell activation, degranulation, and histamine release that can result in severe angioedema and cardiovascular collapse. More commonly seen in women, it is important to consider this reaction when initiating ASA therapy. Recent studies have investigated the TXA2 receptor, as well as the CRTH2 genes in both of these conditions. In women with AERD, the frequency of the CC/CT genotype of TXA2R −+ 795T > C locus is significantly more prevalent, as is the TT genotype of CRTH2 −466T > C locus (82). Patients with a diagnosis of chronic urticaria (CU) did not have a particular genotype at the CRTH2 −466T > C locus, but CU patients with the TT genotype required a significantly higher dose of oral antihistamines to control their clinical symptoms (83). AIU patients demonstrate a higher frequency of the TT genotype at the TXA2R −4684T > C locus, which may be associated with lower TXA2R expression, potentially contributing to the AIU phenotype (84). Finally, a significant association has also been demonstrated in AIU patients and the C haplotype at that IL18 −607A > C gene locus (85).

ANTIHISTAMINES

Antihistamines are used to relieve itch. In dermatology, they are used for the management of atopy, allergic rhinitis, allergic contact dermatitis, and acute urticaria and CU. Histamine, which is produced and stored in mast cells, is mediated by H1 histamine receptors to produce allergic-type itch. First-generation antihistamines (diphenhydramine, hydroxyzine, chlorpheniramine, cyproheptadine, promethazine are very effective, but because they are lipophilic, they cross the blood–brain barrier and can be overly sedating. Other side effects (weight gain, atropine-like effects, including xerostomatitis, blurred vision, constipation, and dysuria) make it difficult to use them for a long term or at increased doses. Second-generation antihistamines (loratidine, cetirizine, fenofexadine, desloratadine) have similar efficacy, but are much less sedating. This group of newer medications have prodrugs and active drug compounds. Most of the antihistamines are metabolized in the liver by the CYP3A4 enzyme system. Patients with liver disease or who are taking concomitant CYP3A4 inhibitors may have longer plasma half-life and a higher serum concentration of the drug, leading to prolonged side effects. Common CYP3A4 inhibitors used in dermatology include erythromycin, ketoconazole, and itraconazole. Because some of the antihistamines, in turn, can act as CYP3A4 inducers or inhibitors, they, too, can increase (or decrease) the serum concentration of other co-administered medications, leading to potentially serious adverse reactions. Terfenidine, a first-generation H1 blocker and astimazole, a second-generation H1 blocker, when taken with other CYP3A4 inhibitors have the potential to cause life-threatening ventricular arrhythmias, such as torsade de pointes. These two antihistamines are no longer on the market in the United States.

Medications that block type 2 histamine receptors (H2) have not shown efficacy in the management of H1-mediated itch. They are generally prescribed for the treatment of gastric histamine release. Some physicians still use them as adjuvant therapy for urticaria. As they are p450 enzyme inhibitors, they have the potential to increase the serum concentration of other medications with narrow therapeutic ranges (and great side effect profiles), including warfarin, phenytoin, theophylline, and imipramine.

IMMUNOSUPPRESSANTS

Cyclosporin

Cyclosporin (CsA) is an immunosuppressant that is commonly used in the treatment of rheumatoid arthritis and psoriasis, as well as prophylaxis to prevent transplant organ rejection. Unlike many other oral immunosuppressants, it is not cytotoxic, does not suppress bone marrow, and it is not teratogenic (14). It is metabolized by hepatic CYP3A4 enzymes and is excreted through bile and feces; dosage reduction is required in patients with liver insufficiency, whereas it is not required in patients with renal failure or on hemodialysis. CsA prevents inflammation by inhibiting IL-2 production by activated CD4+ T cells. CsA binds to cyclophilin, which inhibits calcineurin binding, thus preventing nuclear factor of activated T cells (NFAT-1) from transcribing cytokines, including IL-2. Gene polymorphisms in CYP3A4 have not shown significant alterations in cyclosporine metabolism, per se, however, co-administration with CYP3A4 inhibitors (including ketoconazole, erythromycin, diltiazem, and progesterone) will significantly increase the serum concentration of CsA, leading to increased immunosuppression and increased risk of side effects. Similarly, CYP3A4 inducers (isoniazid, rifampin, clotrimazole, griseofulvin, dexamethasone, carbamazepine, phenobarbital, and phenytoin) have been shown to reduce serum CsA concentration, requiring higher doses for clinical efficacy (86,87).

The ABCB1 gene (a.k.a. multidrug resistance, MDR-1 gene) encodes a P-gp that both metabolizes and is induced by CsA. In adults, there are no significant clinical differences reported in CsA oral bioavailability, with respect to particular polymorphisms in either the ABCB1 gene or the CYP3A genes (88). However, there is strong linkage disequilibrium between particular polymorphisms, creating common haplotypes consisting of 3435C > T and either 2677G/T or 1236C > T (1). Studies in pediatric patients with endstage renal disease have demonstrated an association between CsA oral bioavailability and specific haplotypes of the ABCB1 gene, including 1236C > T and 2677G > T polymorphisms, as well as the related alleles 1199G > C, 1236C > T and 3435C > T, but only in older than eight years of age (89). Carriers of the variant alleles had a CsA oral bioavailability that was 1.5-times higher than patients with wild-type alleles, suggesting that the PK of CsA is related to age or developmental stage (89). It is not yet determined whether or not it is necessary to test for these ABCB1 allelic variants before initiating CsA therapy, to determine optimal dosing in children over eight years of age.

Interestingly, and importantly, an association has been reported between the donor ABCB1 genotype and CsA nephrotoxicity. Donors with the genotype ABCB1 3435 TT have significantly reduced P-gp activity, and standard CsA dosing was strongly associated with CsA nephrotoxicity (90).

Dapsone

Dapsone [4,4-diaminodiphenylsulfone (DDS)] is an antibiotic/antiprotozoic, used in the treatment of leprosy, malaria, and AIDS-related pneumocystis carinii pneumonia. It acts like other sulfonamides, by inhibiting the synthesis of dihydrofolic acid by competitively binding to the active site of dihydropteroate synthetase. While less understood, dapsone also acts as an anti-inflammatory with antineutrophilic effects, used definitively in the management of dermatitis herpetiformis. Other dermatologic applications have included acne (oral and now, topical formulations), Behcet's disease, bullous, and cicatricial pemphigoid, epidermolysis bullosa acquisita, lupus, pyoderma gangrenosum, subcorneal pustular dermatoses, leukoclastic vasculitis and even spider bites (91). Dapsone is absorbed rapidly from the gastrointestinal tract and metabolized in the liver by either *N*-acetylation or *N*-hydroxylation. In the former, primary metabolic pathway, dapsone is acetylated in the liver by *N*-acetyl transferase (NAT2) to monoacetyl dapsone, which then undergoes glucouronidation to produce water-soluble metabolism for renal excretion. There is significant allelic variability in the NAT2 gene. Patients with NAT2*5 (341T > C; amino acid change of Ile114 > Thr), *6 (590G > A; amino acid change of Arg197 > Gln), *7 (857G > A; amino acid change of Gly286 > Glu), and *14 (191G > A; amino acid change of Arg64 > Gln) polymorphisms tend to be PMs/slow acetylators (92). Patients with the slow acetylator phenotype (approximately 40–80% of Caucasians and 10–30% of Asians) exhibit reduced presystemic extraction (i.e, higher bioavailability) and slower elimination of dapsone, but, for this drug, it does not appear to be particularly relevant in its clinical utility, including dosing or increased risk for side effects, including the hepatotoxicity associated with other NAT2-metablized drugs, including isoniazid.

However, patients with mutations in the hydroxylation pathway are at increased risk of significant toxicity, including methemoglobinemia and hemolytic anemia. *N*-hydroxylation of dapsone into the active metabolite, dapsone hydroxylamine, which is a strong oxidant, causes RBC cell membrane damage and subsequent hemolysis. Also, dapsone hydroxylamine reacts with oxyhemoglobin (Fe2+) to form methemoglobin (Fe3+) and nitrosoarene, which gets reduced to another hydroxylamine by NADPH reductase or glutathione in the RBC. While all patients taking dapsone develop a 15% methemoglobinemia, it is not problematic. Patients with levels below 20% are rarely symptomatic. Side effects include nausea, dyspnea, and tachycardia with levels of 30%, lethargy and loss of consciousness with levels of 55% and death at 70% (93). Glucose-6-phosphate dehydrogenase (G6PD) is an antioxidant enzyme that oxidizes and effectively reduces the serum concentration of dapsone hydroxylamine, thus reducing the risk of adverse events. *N*-hydroxylation occurs via various p450 enzymes, including CYP2C9. The PM phenotype is associated with CYP2C9 *2, *3, *5, *6, *8, and *11, and is seen in 1–6% of Blacks, <1% of Asians, and in 2–6% of Caucasians (92). In PMs, dapsone metabolism is shifted significantly to the *N*-acetylation pathway, significantly increasing the amount of bioavailable dapsone and increasing risk of hepatotoxicity.

Co-administration of dapsone with other p450-metabolized medications can alter dapsone levels and impact risk of adverse events. When concurrently prescribed with rifampin, a CYP2C9 inducer, in the treatment of leprosy, it can result in and 7- to 10-fold decrease in dapsone serum levels. While dosing may require adjustment for the treatment of pneumocystis carinii pneumonia, it does not for leprosy, because dapsone levels still reach the minimum inhibitory concentration (94).

Patients with a genetic mutation associated with G6PD deficiency (usually Blacks, Asians, and patients of Mediterranean descent) have an increased buildup of dapsone hydroxylamine, leading to increased RBC hemolysis and a potentially severe anemia. It is recommended that G6PD level and complete blood count be checked before initiating therapy.

Other adverse events associated with dapsone include agranulocytosis and dapsone hypersensitivity syndrome. Agranulocytosis is an idiosyncratic, unpredictable reaction that is most common in patients with dermatitis herpetiformis, with a 25-fold increased risk compared with other patients being treated with dapsone (95). Dapsone-associated drug hypersensitivity syndrome (fever, rash, eosinophilia, and liver and lymph node involvement) is unpredictable, but is most likely to be related to sulfonamide sensitivity. Risk of sulfonamide hypersensitivity increases for patients who are slow acetylators, possibly because of the slow metabolism of the drug. However, polymorphisms in the genes that encode the drug-metabolizing enzymes have not demonstrated an increase in sulfonamide hypersensitivity (96).

Glutathione deficiency has been hypothesized as related to sulfonamide reactions, particularly in HIV-seropositive individuals. Reactive sulfa metabolites can cause direct cell injury and death in vitro in cells infected with HIV. Glutathione helps protect these cells by preventing the oxidation of hydroxylamine and in the formation of more potentially toxic metabolites. However, a prospective study failed to demonstrate glutathione deficiency in HIV-infected patients who develop hypersensitivity (97).

Interestingly, topical dapsone is not associated with methemoglobinemia, and drug hypersensitivity has not been reported.

Tacrolimus (Protopic)

Tacrolimus ointment is a nonsteroidal anti-inflammatory topical therapy, which is indicated for the treatment of atopic dermatitis in

adults and children older than two years. In dermatology, it is used to treat numerous other inflammatory and autoimmune skin conditions, as well, including alopecia areata, vitiligo, rosacea, perioral dermatitis, and pyoderma gangrenosum. Unlike topical steroids, tacrolimus does not thin the skin or induce tachyphylaxis, and systemic levels remain undetected when used in patients with a fairly intact skin barrier. Patients with Netherton syndrome, an autosomal recessive genodermatosis associated with a mutation in the SPINK5 gene, develop atopy, a specific bamboo shaft-like hair-shaft abnormality and ichthyosis associated with severe skin barrier compromise. Systemic levels of this immunosuppressant can become very high in these patients, and so, topical tacrolimus is contraindicated in these patients (98). Signs of tacrolimus toxicity include the following: confusion, headaches, nausea/vomiting, hallucination, asthenia, agitation, seizures, hyperkalemia, hypomagnesemia, pleural effusions, neurotoxicity, and renal failure.

Tacrolimus is also available in oral and parenteral formulations. It is a potent immunomodulator used to prevent post-transplant organ rejection. Side effects include hypertension, itch, and development of diabetes mellitus type 1 (20% of patients). It is metabolized by the CYP3A4 and 3A5 enzymes. CYP3A5*3, a polymorphism attributed to a splicing defect that completely abolishes enzyme activity is a fairly common allele (90% in Caucasians, 75% in Asians, and 50% in African Americans) (99). It leads to very poor metabolizers. Tacrolimus dosing must be varied accordingly.

Concomitant administration of CYP3A4 inhibitors will increase the serum tacrolimus concentration. These include drugs such as azole antifungal agents, corticosteroids, calcium channel blockers, macrolide antibiotics, and gastrointestinal (GI) prokinetic agents. CYP3A4 inducers can lead to decrease tacrolimus concentrations. These include drugs such as cyclosporine, metronidazole, cimetidine, as well as grapefruit juice.

Tacrolimus is also a substrate and potent inhibitor of P-gp, a product of the ABCB1, multidrug resistance gene. However, allelic polymorphisms in the ABCB1 gene do not appear to have significant clinical effect on serum concentrations and clinical efficacy of tacrolimus in adult and even most pediatric patients (100).

Corticosteroids

Corticosteroids are classic immunosuppressants. They reduce inflammation in atopic skin and lung disease, as well as most inflammatory skin and rheumatologic conditions, and are a first-line antirejection therapy in transplant patients. Once absorbed, corticosteroids bind and activate the glucocorticoid receptor (GCR), which is ubiquitous in the body; subsequent translocation of the GCR to the cell nucleus allows it to bind to the glucocorticoid response elements of multiple genes. Anti-inflammatory effects include NF-κB inhibition, induction of lymphocyte and eosinophil apoptosis, inhibition of phospholipase A2 to reduce prostoglandin synthesis, COX-2 inhibition, as well as vascular effects, including inhibition of angiogenesis, vasoconstriction, and decreased vessel permeability, reducing the effect of histamine and bradykinins (14). All glucocorticosteroids, including cortisone, prednisone, and dexamethasone, are substrates for both the CYP3A gene family and P-gp, encoded by the ABCB1 gene. Limited information is available about the effect of genetic polymorphisms on steroid dosing and clinical effect, including which patients are at increased risk of experiencing adverse reactions and which patients may be at increased risk for steroid dependency.

CHEMOTHERAPEUTICS

Epithelial Growth Factor Receptor–Tyrosine Kinase Antagonists

The activation of epithelial growth factor receptor (EGFR), expressed in the basal layer of normal skin, is a key factor in epithelial cell production, proliferation, as well as normal and tumor cell motility, adhesion, invasion, survival, and angiogenesis. EGFR is a transmembrane cell surface receptor tyrosine kinase (TK) that, when dimerized, leads to the activation of cytoplasmic TK activity, leading to the autophosphorylation of the intracellular domain of EGFR, and activation of downstream signaling pathways (e.g., Ras/mitogen-activated protein kinase pathway and the PI3K/Akt pathway and Janus-activated kinase signal transducers and activator of transcription) that are involved in tumor growth and metastasis. The reversible EGFR–TK inhibitors, such as gefitinib and erlotinib, prevent autophosphorylation of the receptor, thus inhibiting tumor growth and metastasis. They have been used in the treatment of metastatic basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), although these are not yet US FDA-approved indications. Treatment with gefitinib or erlotinib results in dramatic clinical response in approximately 10–30% patients with non–small cell lung cancer (NSCLC). It is agreed that specific somatic mutations of the EGFR gene confer sensitivity or resistance to EGFR–TK inhibitors.

The most prevalent activating EGFR kinase domain mutations in NSCLC include in-frame deletions in exon 19 (accounting for 44% of EGFR mutations), an L858R substitution in exon 21 (accounting for 41% of EGFR mutations), nucleotide substitutions in exon 18 (e.g., G719C or G719S) (5%), and in-frame insertions in exon 20 (<1%). The most noteworthy clinically relevant mutations associated with resistance to EGFR–TK inhibitors include T790M in exon 20, and D761Y, a T790M-like secondary mutation in exon 19 (101).

Activating EGFR–TK mutations is significantly more common in East Asians, women, never smokers, and patients with adenocarcinoma (1). Interestingly, this mirrors the clinically defined subsets of patients who were most likely to respond to EGFR–TK inhibitors. NSCLC patients who present with EGFR-TK–activating mutations have not only better response to gefitinib or erlotinib but also significantly longer progression-free survival and overall survival compared with those without these mutations (1). Furthermore, frequent and severe papulopustular eruptions (seen in 45–100% of patients receiving EGFR inhibitors) is associated with increased tumor response and longer median survival, so cessation of treatment because of this side effect is not recommended (102). The rash can be managed with oral antibiotics and low-dose retinoids. Other cutaneous side effects commonly seen with these medications include scalp/body alopecia, trichomegaly, facial hirsuitism, xerosis, pruritus, and nail changes (onychoschesis, paronychia, periungual pyogenic granulomas) (103).

Vemurafenib (PLX 4032; Zelboraf)

Targeted therapy for unresectable or metastatic melanoma is the current therapeutic trend, and recently, there have been some major breakthroughs. Most research has focused on the MAP Kinase (including RAS, n-RAF, b-RAF, MEK, and ERK) and Pl3 Kinase pathways. The MAPK pathway is constitutively activated

in many melanomas; blockade at specific steps likely prevents phosphorylation and activation of transcription factors, thus preventing cell growth and proliferation.

Almost 70% of human melanomas have somatic activating mutations in the b-RAF proto-oncogene, specifically involving the single substitution, V600E. Highly selective b-RAF kinase inhibitors that target this signature polymorphism have been under development. Sorafenib (Bayer), one of the first agents to target molecules, including b-RAF kinase, demonstrated no clinical effect on melanoma (104). However, US FDA-approved PLX 4032, now known as vemurafenib (Zelboraf, Genentech USA, Inc., 1 DNA Way, South San Francisco, CA, USA), which specifically targets the BRAF-V600E mutation, demonstrated tumor regression in 81% of patients in the initial study of 32 patients (105,106). A more recent phase III trial of 675 patients found significantly better response rates in patients treated with vemurafenib compared with patients treated with dacarbazine (six-month survival rates were 84% and 64%, respectively). The authors calculated a relative reduction in mortality of 63% and of either death or disease progression of 74% compared with standard dacarbazine treatment (107).

Although some drug resistance has begun to emerge (108), it is a promising, personalized treatment modality.

Ipilimumab (MDX-010; Yervoy)

With an immune-blockade goal, similar to current cytokine therapy (IFN-alpha, IL-2), the US FDA has also approved an anti-CTLA-4 therapy, which blocks the negative co-stimulatory molecule on T cells, thus enhancing T-cell activation and augmenting the patient's antitumor immune response. Ipilimumab (MDX-010, Bristol-Myers Squibb Company Princeton, NJ, USA), a human monoclonal antibody, has shown efficacy in late-stage, unresectable metastatic melanoma (109). An randomized controlled trial involving 676 patients, which compared survival with ipilimumab plus a tumor vaccine, ipilimumab plus a placebo vaccine, and vaccine plus placebo drug demonstrated prolonged survival (median of four months) in patients treated with ipilimumab alone compared with vaccine alone ($P = 0.0026$) and also by a median of four months in patients treated with vaccine plus ipilimumab compared with vaccine alone ($P = 0.0004$) (110).

Imatinib (Gleevec)

Mucosal and acral melanomas tend not to have b-RAF mutations. Rather, 30–40% of them have activating c-kit mutations (111). Imatinib (Gleevac, Novartis Pharmaceuticals Corporation East Hanover, New Jersey, USA), a c-kit inhibitor, is being investigated for targeted therapy for these melanomas. Some smaller studies have shown both partial and dramatic responses in certain subgroups of patients (112), but further investigation is needed to determine how effective c-kit inhibitors can be in melanoma management.

Imatinib has proven effective in the treatment of dermatofibrosarcoma protuberans (DFSP), which, albeit rare, is, the second most common cutaneous sarcoma. With an incidence of 4.5 per 1 million persons, it is a slow-growing tumor, usually of the head and neck. It has up to 60% local recurrence rate, but fewer than 5% of patients with DFSP develop metastatic sarcoma, which has a poor prognosis. DFSP develops from a genetic translocation between chromosomes 17 and 22 [t(17;22)(q22;q13)] that fuses the promoter of the collagen gene COL1A1 to the platelet-derived growth factor beta-chain gene PDGFB, causing an overproduction of PDGF locally, and promoting autocrine or paracrine tumor growth (113,114). Imatinib was developed as an inhibitor of the PDGF receptor TK and has proven clinical activity against chronic myelogenous leukemia (expressing bcr-able, a fusion product from the t(19;22) translocation) and gastrointestinal stromal tumors (expressing c-kit). In unresectable, recurrent and metastatic sarcomas, targeted gene neoadjuvant therapy with imatinib has shown success. Imitanib, a c-kit inhibitor, induces apoptosis and tumor remission in tumors with this t(17;22) translocation (115,116). Adverse effects of Imitanib therapy in DFSP patients, include TEN, acute generalized eruptive pustulosis and DRESS (117).

Vismodegib (GDC-0449)

Ultraviolet light (UVL)-induced epidermal DNA damage is thought to be the primary event in the development of BCCs. Specific genodermatoses associated with the development of multiple BCCs from a young age are often associated with a genetic inability to repair this DNA damage. In basal cell nevus syndrome (BCCNS), the predisposition to many, early, sporadic BCCs involves a mutation in the patched gene (PTCH), a component of the hedgehog signaling pathway that normally encodes a primary inhibitor of hedgehog signaling; this mutation turns on the smoothened gene, and keeps the pathway constantly signaling, which can lead to cell proliferation and tumor growth. This same pathway is involved in many UVL-induced BCCs. In patients with unresectable, locally aggressive or metastatic disease (118), or, particularly, in BCCNS patients who can develop hundreds of BCC tumors, targeted molecular therapy that blocks the hedgehog pathway from signaling can prevent and treat these BCCs

GDC-0449 (Vismodegib, Patheon, Inc., Mississauga, Canada) is an orally administered molecule that binds the smoothened receptor, thus preventing hedgehog signaling. After a phase I study of 33 adult patients with aggressive, nonresectable BCCs demonstrated tumor regression and clearance with vismodegib (118), Dr Tang and associates examined the efficacy of this treatment in patients with BCCNS (119).

In their randomized, controlled phase II study of 41 patients with BCCNS, the number of new surgically eligible BCCs that developed was 0.07/month in patients receiving active treatment with vismodegib (GDC-0449), compared with 1.74/month in those who received placebo ($P < 0.0001$). The change from baseline in the aggregate size of existing BCCs was 24 cm in the vismodegib group, compared with 3 cm in the placebo group ($P = 0.006$). In addition to preventing new skin cancers, treatment with vismodegib typically began shrinking existing tumors within the first month or two. Sixty percent of biopsied tumors that appeared clinically clear showed histologic clearance.

Interestingly, these same patients had a clinical improvement in other cutaneous features of the syndrome, including palmar pits. This medication does prevent the need for multiple, disfiguring surgeries in patients with BCCNS; however, in healthy patients with only a few UVL-induced tumors, side effects of vismodegib prevent this from being a universally beneficial treatment. These include taste loss (in 83% of patients on treatment *vs* 8% on placebo), muscle cramps (in 67% *vs* 8%), and weight loss (in 50% *vs* 8%). Hair loss also was common. The authors of the study suggest that this treatment be employed for 6–12 months every few years to reduce the number of BCCs on the skin.

Celecoxib

Nonsteroidal anti-inflammatory drugs (NSAIDs) have shown some efficacy in reducing tumor burden, particularly in gastrointestinal tumors. Blockade of the prostaglandin metabolic pathways, regulated by the cyclooxygenase enzymes (COX-1, COX-2) may prevent tumor formation.

A double-blind placebo-controlled randomized trial involving 240 healthy subjects 37–87 years of age with 10–40 actinic keratoses was conducted at eight US academic medical centers (120). Patients were randomly assigned to receive 200 mg of celecoxib or placebo administered orally twice daily for 9 months. Subjects were evaluated at 3, 6, 9 (ie, completion of treatment), and 11 months after randomization. The primary endpoint was the number of new actinic keratoses at 9 months; the number of nonmelanoma skin cancers combined and SCCs and BCCs separately per patient at 11 months was assessed, as well. There was no difference in the incidence of actinic keratoses between the two groups at 9 months after randomization, whereas at 11 months after randomization, there were fewer NMSCs in the celecoxib arm than in the placebo arm [mean cumulative tumor number per patient 0.14 *vs* 0.35; rate ratio (RR) = 0.43 (for only BCCs, RR = 0.40), 95% confidence interval = 0.24–0.75; $P = 0.003$]. With the occurrences of serious and cardiovascular adverse events similar in the two groups, celecoxib may be effective for prevention of SCCs and BCCs in individuals who have extensive actinic damage and are at high risk for NMSC.

In patients with BCCNS, who have a germline mutation in the patch gene (PTCH1), constant signaling of the hedgehog pathway leads to tumor formation. Dr Tang and associates demonstrated that the incidence of BCCs in mice with a mutated PTCH1 gene is associated with overexpression of COX-1 or COX-2. They were able to demonstrate that genetic deletion of COX-1 or COX-2 decreased the microscopic tumor burden by 75% in these mice. However, pharmacologic inhibition with celecoxib only reduced the tumor burden by 35%.

A second phase of their study was designed to determine the efficacy of celecoxib in inhibiting genetically induced BCC in patients with BCCNS. Study patients who had less than 15 active BCCs at baseline showed a reduction in BCC development over the 3-year study period. Those receiving placebo had a 50% increase in BCC count per year, whereas those receiving celecoxib had only a 20% increase. This finding was not reproducible in patients with greater than 15 BCCs at baseline. Despite the paucity of related serious adverse events, including no reports of cardiovascular or cerebrovascular accidents, there is still the stigma of cardiovascular events in patients taking COX inhibitors (other natural molecules known to inhibit COX without the cardiovascular risk). These may prove effective in the management of tumor burden in BCCNS (121).

ANALGESICS/ANESTHETICS

Eutectic Lidocaine and Prilocaine Cream

Topical anesthetics are a simple, convenient method for reducing pain in the skin. US FDA approved use on normal, intact cornified skin. It is used to facilitate venipuncture, laceration repair, laser treatment, wound and ulcer debridement, as well as pain management, including mucosal ulcers, postherpetic neuralgia, and pruritus. Moreover, it has become a common practice in pediatric patient care. Eutectic lidocaine and prilocaine (EMLA) cream is applied for 60–120 minutes under occlusion to yield an up to 5 mm depth of anesthesia for up to 1 hour once removed. While the prilocaine makes it more effective than topical lidocaine alone, it is associated with an increase risk of methemoglobinemia (metHb). Although this is a risk in all pediatric and adult patients, the risk is significantly increased in neonates, due to the immaturity of the metHb reductase pathway (122), their increased sensitivity to the oxidizing effects, as well as their already reduced level of normal oxygen-carrying adult hemoglobin (Hb), because of their still-high levels of fetal Hb. The manufacturers (AstraZeneca LP, Wilmington, DE, USA) recommend that EMLA not be used in neonates younger than 37 weeks gestational age, in infants younger than 12 months being treated with medication that can also induce metHb (acetaminophen, sulfonamides/sulfones, phenytoin, phenobarbital, nitroglycerin, nitrous oxide, antimalarials), or patients with a congenital or idiopathic metHb (14). Because topical and local anesthetics can cross the placenta, newborns can be born with metHb, if they are used during labor and delivery. While EMLA is generally considered safe for neonatal circumcision, venipuncture, and even management of ulcerated vascular lesions, and metHb is not common, it is important to note that antidote (methylene blue) has a very small, safe therapeutic window and is toxic at only slightly higher doses (123).

Opioids

Adequate pain management is the bane of many medical practitioners in the United States. Adjuvant pain medications, including NSAIDs are rarely sufficient for moderate or severe pain, and higher doses are associated with unacceptable side effects, including gastrointestinal bleeds, and increased risk of myocardial infarction and stroke (124). The World Health Organization (WHO)'s standardized, ladder -approach to pain management recommends opioids as the next step. Opioids are narcotic drugs that are intended to provide analgesia, but when bound to the mu (μ) opioid receptor, too high doses can produce side effects, including euphoria, miosis, sleep, stupor, coma, respiratory depression, and death. The problem is determining what is too high a dose. Standard dosing is used for all patients at baseline. A specific analgesic effect is expected. When patients do not respond as predicted, it is often assumed that they are noncompliant, have developed drug tolerance from previous opioid administration, or are exhibiting addictive, drug-seeking behavior. The impact of an individual's genetic heterogeneity at various points in the drug (or prodrug)'s metabolism, as well as the genetic variability in the number and functional status of the drug receptors, can help explain the level of analgesic effect and side effect tolerance.

Codeine, the most common opioid prescribed, is metabolized similar to oxycodone, hydrocodone, tramadol, propoxyphene, and methadone. First, 90% of the dose is metabolized to inactive compounds by CYP3A4. The remaining 10% is then metabolized to active morphine compounds by CYP2D6, in a process called O-demethylation (125). Allelic variants in the CYP2D6 categorize patients as PMs, IMs, EMs, and ultra-rapid metabolizers (UMs). EM is considered the normal variant and homozygous for two copies of the wild-type allele. PMs have two deficient alleles (*3, *7) and are unable to metabolize most opioids, as well as most antidepressants and antipsychotic medications. They have a high level of prodrug in their blood, but will experience no analgesia from standard or higher doses. In fact, EMs will have a five

times greater serum concentration of active drug (e.g., hydromorphone) than PMs, after administration of a single 10 mg dose of hydrocodone (126). PMs are found in 7–10% of the Caucasian population and in 1–2% of the African American population. UMs differ in that they carry three or more wild-type, fully functional alleles (*1xN, *2xN). Because they metabolize drug so quickly, they often "miss" the therapeutic effect of standard dosing, or experience dangerous, even toxic side effects because of the buildup of active drug (morphine). Because of reported deaths in newborns of breastfeeding UM mothers who have received codeine for postpartum pain management, the US FDA has amended drug inserts to include a warning for UM mothers, guidelines for the recognition of morphine toxicity and identification of CYP2D6 UMs (127).

Opioid receptors, mu (μ) in particular, are G-coupled transmembrane proteins that mediate the analgesic effect of opioid drugs. There are at least seven spice variants of the OPRM1 μ receptor gene that affect the binding and analgesic effect of certain opioid drugs, including morphine, hydromorphone and fentanyl. The most common is the 118A > G single nucleotide polymorphism of exon 1 of OPRM1 (renamed 304A/G10), which creates a missense mutation through the substitution of an asparagine for aspartic acid at the N-terminus, resulting in the loss of one of five essential *N*-glycosylation sites on the receptor (1,125). This allelic variant occurs in 10–40% of the population, depending on ethnicity (1). OPRM1 GG patients require significantly higher opioid dosages to achieve comparable pain relief to AA patients. Two recent studies in pain and cancer patients demonstrated that when OPRM1 AA patients were administered 112 mg/24 hours or 97 mg/24 hours, respectively, comparison OPRM1 GG patients required 216 mg/24 hours or 225 mg/24 hours, respectively, to achieve similar analgesia. The use of CYP2D6 and OPRM1 gene polymorphisms as biomarkers may help detect pain patients who require individualized opioid therapeutic regimens to optimize pain relief and prevent toxic side effects.

The majority of classical analgesics target OPRM1 and PTGS2, as well as signaling pathway genes, including KCNJ6 (128). New analgesics are being developed to target genes associated with voltage and ligand-gated sodium, calcium, and potassium channels, specifically SCN9A, CACNA1B, KCNQ2, and KCNQ3. Allelic mutations in voltage-gated transient receptor potential channel genes (TRPV), including TRPV1 and 3 are already demonstrating reduced analgesic efficacy. Cannaboid receptor genes (CNR1, 2), bradykinin B1 (BDKRB1), 5-HT (HTR1A), and nerve growth factor genes (NGFB) are currently being investigated as possible targets for new analgesics (128).

CONCLUSION

The consideration of pharmacogenetics in the attempt to understand how drugs are metabolized and how they clinically affect patients has heralded a new age of individualization of drug therapy. We have been aware of families of drug metabolizing enzymes, including the p450 family, and the clinical effects that various co-administered drugs have in inducing and inhibiting the anticipated effects of medications that are metabolized by that particular enzyme. Recognition of specific allelic variations in some enzyme-encoding genes can help determine if and how effective a drug may be for an individual patient, and for whom this drug may induce significant adverse events. These genetic polymorphisms can be used as biomarkers. For many current medications and, surely for many more in the future, genetic testing of patients for drug-specific biomarkers before initiating a standard therapy can be used to predict clinical response, thus decreasing the frequency of toxic, adverse events, as well as optimizing treatment efficacy.

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2 Hormesis and dermatology

Audris Chiang, Haw-Yueh Thong, and Howard I. Maibach

INTRODUCTION

Biphasic dose response, namely a low-dose stimulatory and a high-dose inhibitory response, also called hormesis in the field of toxicology, has been noted in a wide range of biological model systems from immunology to cancer biology (1–4). Calabrese (1–4) has been the mainstay in bringing the attention of the scientific community to this interesting and a not uncommon phenomenon. As noted by Calabrese, the quantitative features of the hormetic-like biphasic dose response were remarkably similar with respect to the amplitude of the stimulatory response, the width of the stimulation, and the relationship of the maximum stimulatory response to the zero equivalent point (ZEP, ie, threshold). Typically, the low-dose hormetic biphasic dose–response stimulation is modest, with maximum stimulation between 30 and 60% greater than controls, and has a rather similar appearance in different cell types with various chemicals (4). Most stimulatory ranges were less than 100-fold (averages 10- to 20-fold) measuring back from the ZEP. The low-dose stimulatory response often occurs following an initial disruption in homeostasis and appears to represent a modest overcompensation response. It is believed that the modest stimulatory responsiveness is due to a compensatory process that "slightly" overshoots its goal of the original physiologic setpoint, ensuring that the system returns to homeostasis without unnecessary and excessive overcompensation (5). Therefore, it is important to follow the dose–response relationships over time to better define its quantitative features. Although initial interest focused on the hormetic effects of pollutants and toxic substances on biological systems (6), the interest expanded to include pharmacologic agents, phytocompounds, as well as endogenous agonists (4). The hormetic-like biphasic dose–response relationships appear to be highly generalizable; that is, such responses do not appear to be restricted by biological model, endpoint, or chemical/physical stressors (4).

Many investigations attempted to assess mechanisms that could account for the hormetic-like biphasic dose–response relationship. In general, there is no single mechanism that accounts for the plethora of hormetic relationships. Nonetheless, a common molecular tactic by which biphasic dose–response relationships are displayed involves the presence of two receptor subtypes affecting cell regulation, one with high and the other with low affinity for the agonist but with notably more capacity (ie, more receptors) (4). Such an arrangement may lead to the biphasic dose response, with the high-affinity receptor activated at low concentrations, which stimulates DNA synthesis and cellular proliferation; and the low-affinity/high-capacity receptor becoming dominant at higher concentrations decreasing the cell proliferative response. This is a general pharmacologic mechanism in that it is used for a large number of receptor-based responses from cancer cells to neutrophil chemotaxis and many others.

This chapter reviews hormetic effects of various agents on skin biology. Recognition of this emerging biological phenomenon in dermatology should lead to markedly improved integrative assessments of animal/human skin responses to toxic substances, pharmacologic agents, and endogenous agonists.

EVIDENCES OF HORMESIS IN SKIN

Skin is a complex biological model but highly approachable. Models exist for dermatologic research, which include animal *versus* human skin models, in vitro *versus* in vivo models, regional variation, stem cell biology, and hair follicle biology. Many pharmaceutic preparations in dermatology affect cell regulation. Nonetheless, the US Food and Drug Administration sometimes exempts dose justification for dermatologic preparations. As a result, the presence of any hormetic effect might have been missed.

The literature in dermatology indicates that several cell types in the skin provided evidence of hormetic-like biphasic dose/concentration–response relationships. A brief listing of the cell types showing hormetic relationships and the quantitative features of dose responses is presented in Table 2.1.

MELANOMA AND TUMOR CELL LINES DISPLAY HORMETIC DOSE RESPONSES

Perhaps a more important issue regarding hormesis is its relationship to cancer biology. The existence of hormetic dose responses in many tumor cell lines has been noted and reviewed by Calabrese (4). Twelve melanoma cell lines (M4Beu, B16, M24, MNT, SK-MEL, H1144, SK-MEL28, Cal 1, Cal 4, Cal 23, Cal 24, and Cal 32) have been shown to display hormetic dose responses to various chemicals (guanine or guanosine derivatives, mistletoe extract, salsolinol, tetrahydropapaveroline, dopamine, resveratrol, thrombin, and suramin). Numerous endogenous agonists, drugs, environmental contaminants, and phytochemicals, some relevant to dermatotoxicology and dermatooncology, have also been noted to exert hormetic dose responses in various tumor cell lines (4). Examples and the proposed mechanistic explanations are listed in Table 2.2.

DISCUSSION

Calabrese and Blain (40) developed a hormesis database, containing 5600 hormetic-like dose–response relationships over approximately 900 agents from a broadly diversified spectrum of chemical classes and physical agents, stressing the general robustness of published studies to establish support for the hormetic

TABLE 2.1
Examples of Hormesis in Skin

Chemicals	Cell Type	Stimulatory Responses	Inhibitory Responses	References
SLS	Cultured keratinocytes	SLS (10^{-8} to 10^{-5} M): For 1 h: 36% stimulation For 18 h: 12% stimulation For 4 days: ~89% stimulation	$>10^{-5}$ M	(7)
	Cultured fibroblasts	Subconfluent fibroblast: For 1 h (10^{-5} M): 38% stimulation For 18 h (10^{-6} M): 32% stimulation Confluent fibroblast for7op[]\ 1 h (10^{-8} M): 40% stimulation		
SLS *versus* RA	Cultured keratinocytes	0.1–1 × 10^{-5} M: 1. For 2 h: from 4 × 10^{-4} (baseline cell count) to a maximum of 8 × 10^{-4} cells 2. Less potent than RA at 0.75–3 × 10^{-6} M	>5 × 10^{-5} M	(8)
	Cultured dermal fibroblasts	1. Similar stimulatory response as keratinocytes at the same doses 2. Less effective than RA in stimulating production of extracellular matrix		
Imidazole derivatives (econazole, clotrimazole)	Reconstructed human epidermis	5–133 µM (18 h contact time): Biphasic effect on 7-ethoxycoumarin-*o*-deethylase activity in the epidermis, with induction (60–70% increase of the basal level) at low concentrations and inhibition (40% of the basal level) at high concentrations	>133 µM	(9)
Ionizing radiation	Cultured human dermal fibroblasts	The plating efficacy reached values significantly above 100% apparent survival at low doses (≤40 cGy)	≥40 cGy	(10)
Corticotropin-releasing hormone	Cultured human sebocytes	Biphasic effect on sebaceous lipid synthesis and upregulation on the mRNA levels of 3-β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (10^{-7} M), but did not affect cell viability, cell proliferation, or IL-1β-induced IL-8 release		(11)
Arsenite	Human epidermal keratinocytes, promyelocytic leukemia cells	Exposed to arsenite from 0.1 to 40 µM for 1, 3, and 5 days, cell growth was increased at low doses (0.5 µM)	>1 µM	(12)
Arsenic trioxide (As)	Keratinocytes, melanocytes, dendritic cells, dermal fibroblasts, microvascular endothelial cells, monocytes, T cells	Exposed to for 72 h: 1. Sublethal doses of As stimulate cell proliferations 2. As is toxic at high doses to keratinocytes, fibroblasts, monocytes, and T cells; and toxic at low doses to melanocytes, microvascular endothelial cells, and dendritic cells Peak proliferation: Keratinocytes: 30 µM; melanocytes: 0.95 µM; dendritic cells: 0.96 µM; fibroblasts: 7.6 µM; microvascular endothelial cells: 0.95 µM; monocytes: 30 µM	LD_{50}: Keratinocytes: 45.5 µM; melanocytes: 7.6 µM; dendritic cells: 7.6 µM; fibroblasts: 187 µM; microvascular endothelial cells: 2.4 µM; monocytes: 252.7 µM	(13)
Mixture of four metals (arsenic, chromium, cadmium, lead)	Human keratinocytes	Exposed for 24 h: Lowest mixture dilution (0.0014× of As 7.7 µM, Cr 4.9 µM, Cd 6.1 µM, Pb 100 µM)) with a total concentration of 0.163 µM had a percent viability of 116.6%, clearly above that observed in the single-chemical data. It is likely that this enhancement of cell viability at the lowest mixture level is indicative of the presence of hormesis	Synergistic cytotoxicity at total concentration of 8–36 µM of the metal mixture	(14)
Arsenite (iAs^{III}), arsenate (iAs^{V}), methylarsine oxide ($MAs^{III}O$), complex of dimethylarsinous acid with glutathione ($DMAs^{III}GS$), methylarsonic acid (MAs^{V}), dimethylarsinic acid ($DMAs^{V}$)	Human keratinocytes	1. iAs^{III} and $DMAs^{III}GS$ induced an increase in cell proliferation at low concentrations (0.001–0.01 µM), while at high concentrations cell proliferation was inhibited 2. Pentavalent arsenicals did not stimulate cell proliferation 3. Methylated forms of As^{V} were more cytotoxic than iAs^{V}	iAs^{III}: >0.5 µM; iAs^{V}: >1.0 µM; $DMAs^{III}GS$: >0.5 µM; $DMAs^{V}$: >0.1 µM	(15)

Minoxidil	Human epidermal and follicular keratinocytes	0.1–10 µM (exposed for 5–8 days): Minoxidil had biphasic effects on the proliferation and differentiation of both epidermal and follicular keratinocytes, stimulating proliferation at micromolar doses, while antiproliferative, prodifferentiative, and partially cytotoxic effects were observed with millimolar concentrations	>1 mM	(16)
NO donors: Nitroprusside, SIN-1, DETA/NO, SNAP	Human keratinocytes, fibroblasts	Four different NO donors at concentrations ranging from 0.01 to 5 mM were added every 12 or 24 h, and cells cultured for up to 3 days in the presence of these compounds. Keratinocytes: 1. A biphasic effect is found with increased proliferation at low concentrations and cytostasis at high concentrations 2. Cytokeratin 6 expression is decreased at the lower NO donor concentrations and increased at higher concentrations as an indication of induction of differentiation at higher NO concentrations Fibroblasts: Cytostasis becomes significant at ≥0.25 M of the NO donor		(17)
Antioxidants: Asc2P, Asc2G Prooxidants: Hydrogen peroxide (H_2O_2)	Human keratinocytes	Repetitive addition of Asc2P and Asc2G: Cellular life-span of keratinocytes was shown to be extended up to 150% of population doubling levels (PDLs) Prooxidants: 20 µM H_2O_2: extended up to 160% of PDLs 60 µM H_2O_2: extended up to 120% of PDL		(18)
1,25-Dihydroxy vitamin D_3 ($1,25(OH)_2D_3$)	Whole organ cultures of hair follicle	Biphasic dose–response relationships for the effects of $1,25(OH)_2D_3$ on the total cumulative growth of hair follicles and hair fibers At relatively low concentration, growth of follicles and fibers was stimulated, to a maximal extent at 10 nM of 52 and 36% The concentration producing 50% of the maximal response (EC_{50}) for both follicle and fiber growth stimulation was 0.3 nM The increase in cumulative growth was due to stimulation of the initial, linear growth phases	Dose-dependent and complete inhibition of follicle and fiber growth at 100 nM	(19)
CPFX	Human fibroblasts	The effect of CPFX on cell viability is time dependent: 1. CPFX was not cytotoxic at any concentration when the cells were incubated for 24 h 2. Low concentrations (0.0129 and 0.032 mM) of CPFX increased the cell survival in all incubation periods tested	Decreased viability was observed at 0.129 and 0.194 mM (48 h of exposure), and 0.129 M (72 h of exposure)	(20)

Abbreviations: Asc2P, Ascorbic-2-*O*-phosphate; Asc2G, ascorbic-2-*O*- alpha-glucoside; NO, nitric oxide; RA, retinoic acid; SLS, sodium lauryl sulfate; CPFX, ciprofloxacin.

TABLE 2.2
Examples of Dermatology-Relevant Chemicals Displaying Hormetic Dose–Response Relationship in Tumor Cell Lines

Chemicals	Tumor Cell Lines	Possible Mechanisms	References
Endogenous agonists			
EGF	Ovarian, colon, epidermoid, breast	A431 cells: 1. The dual effect (stimulation/inhibition) of EGF on its proliferation is associated with differential pattern of MAP kinase activities, which may involve the action of specific phosphatase(s) 2. Dependent on the quantity of occupied EGF-R: A critical and restricted number of sites are involved in EGF growth stimulation 3. Low-dose stimulation is mediated by a minority population of high-affinity EGF-Rs	(21–24)
Estrogen	Colon, breast	Colon cancer cells: Physiological concentrations of estradiol acting via the classical ER may have a proliferative effect. When there are high luminal concentrations of estrogenic compounds, they may act on low-affinity estrogen binding sites that mediate the growth-inhibitory effect	(25)
Progesterone	Ovarian	HOSE and Oca cells: Stimulation by progesterone at low concentrations, marked inhibition at high concentrations, both blocked by specific progesterone antagonist, confirming the specificity of the hormonal action	(26)
Phytocompounds			
Daidzein	Breast	1. Isoflavones elicit a biphasic response in the DNA synthesis and cell proliferation of the ER of positive human breast cancer cells 2. Effects of diadzein and biochanin A on these cells appeared to be associated with the expression of P53	(27)
Genistein	Colon, breast, oral	1. Binds to the ER at estrogen-binding site; the formed complex then interacts with the ERE1, thereby promoting the transcription of estrogen-regulated genes 2. MCF-7 cells: Cell proliferative effects were mediated through ER, while antiproliferative effect was independent of ER	(28,29)
Glabridin	Breast	Proliferation of ER^+ cells was highly associated with the binding affinity of glabridin to the ER. Optimal cell proliferation occurred at a concentration at which half of the ER sites were saturated	(30)
Quercetin	Breast, oral	1. Similar to genistein, a biphasic effect on cell proliferation with ER involvement 2. Regulatory overcorrections by biosynthetic control mechanisms to low levels of growth inhibiting challenge 3. Concentration-dependent antioxidant and prooxidant activities	(31)
Resveratrol	Breast, leukemia	1. MCF-7 cells: At low concentration, acts as a partial ER agonist. At high concentrations, causes inhibition of MCF-7 cells regardless of ER status, possibly via the antagonizing of linoleic acid (a potent stimulator of breast cancer cells)	(32)
Drugs			
Dexamethasone	Neuroepithelial, pancreas, meningiomas	1. Brain tumor: Low-dose stimulation is related to the presence of glucocorticoid receptor (probably necessary but insufficient). Inhibitory effects at high doses were believed not to be due to receptor mediation but by other mechanisms, such as cell membrane alterations 2. Neuroepithelial cancer cells: a. Dexamethasone treatment causes glucocorticoid receptors translocation into the nucleus to modulate cell proliferation upon binding of different concentrations of dexamethasone. Dexamethasone inhibits proliferation of some neuroepithelial cell lines, not by glucocorticoid-induced apoptosis b. Lower concentrations of dexamethosone stimulate growth only in glucocorticoid-positive tumors, suggested the role of the specific receptor. Higher concentrations inhibit cell growth not due to receptor mediation, but seems to be related to other mechanisms (cell membrane alterations)	(33–35)
Retinoic acid	Breast, prostate, glioblastoma	1. Breast MCF-7 cells: via ICF-1 receptor: Lowering ICF-1 levels inhibits cell proliferation 2. Prostate LNCaP cells: Possible roles of retinal-binding proteins and retinoic receptors, which may have biphasic mitogenic effects on LNCaP cells and are concentration-dependent in affecting prostate specific antigen secretion	(36,37)
Toxic substances			
Cadmium chloride	Ovarian	No cytotoxicity at low concentration, but has stimulatory effects on metabolic activities particularly in mitochondria via unknown mechanism	(38)
Sodium butyrate	Colon	Dependent on the other energy sources available to epithelium: In conditions of low-energy availability, butyrate could be both stimulatory/trophic. In the presence of high levels of alternative energy sources, such as glucose, butyrate could inhibit growth/induce apoptosis	(39)

Abbreviation: EGF, epidermal growth factor.

dose–response hypothesis. Table 2.1 showed that clear examples of hormesis do exist in dermatology, and Table 2.2 suggested that the presence of hormesis in cancer biology may be an important phenomenon not to be overlooked.

Despite the extensive observation of hormetic dose–response relationships for numerous agents across the biological spectrum, most studies assessed cellular responses. Few studies followed up in animal and human models—normal or disease—assessed the simultaneous responses of different systems to the same agent. We believe in vivo studies are necessary to provide an integrative assessment of the whole animal/human responses to various agents, to document any discrepancies between the in vitro and in vivo responses, and to clarify the clinical implication of hormesis.

Studies on the mechanism of action and the exact definition of the low dose to be applied are essential to achieve a better understanding of hormesis. Another important issue to discuss in the field of hormesis, as proposed by van der Woude et al. (41), is the need for risk assessment paradigms to be modified to take hormesis into account. Rietjens and Alink (42) also suggested that more focus should be redirected from looking only at adverse effects at high levels of exposure to characterizing the complex biological effects, both adverse and beneficial, at low levels of exposure. Low-dose toxicology and pharmacology will contribute to better methods for low-dose risk assessment of chemical compounds and their effect on carcinogenesis, taking into consideration that the ultimate biological effect of a chemical may vary with its dose, the endpoint or target organ considered, cellular interactions, and the combined exposure with other chemicals.

Evidence from other studies have shown that keratinocytes and fibroblasts exposed to hormetic mild stress treatments, such as repeated mild heat shock, displayed beneficial antiaging effects (43–45). Antiaging effects, such as reduced accumulation of damaged proteins were brought about by stimulation of production of heat shock proteins from the mild heat shock treatments, where these heat shock proteins functioned in pathways involved with refolding or degrading damaged proteins (46).

We believe skin is an excellent candidate to gain entrance into this biology due to its accessibility; its complex nature, with highly differentiated cell types and various subsystems (keratinocytes, melanocytes, Langerhans cells, fibroblasts, epidermis, dermis, hair follicle, eccrine, apocrine, and sebaceous units); and the availability of specialized noninvasive technology for in vivo studies (47,48). In addition, skin has been among the first organs analyzed using DNA microarrays in various topics from skin cancers, melanomas, basal cell carcinomas, squamous cell carcinomas, psoriasis, and other inflammatory disorders, to stem cell biology, the biology of epidermal keratinocytes, and so forth (Table 2.3) (49). DNA microarray studies will be an excellent tool to elucidate the mechanisms of hormesis in skin biology. In short, better understanding of hormesis will probably lead to different strategies for risk assessment process employed in the fields of dermatologic toxicology and pharmacology.

CONCLUSION

Hormesis is a common phenomenon in dermatology and other fields. Detailed consideration should be given to its concept, its risk assessment implications, and its clinical significance. However, without additional mechanistic insight, the consequences of hormesis for risk assessment and the possibilities for in vitro to in vivo extrapolation will remain limited.

TABLES 2.3
Targets for DNA Microarray Studies in Dermatology and Skin Biology (49)

Melanoma and melanocytes
Carcinomas (basal cell carcinoma, squamous cell carcinoma)
Keratinocyte differentiation
Wound healing and inflammatory diseases
Proinflammatory and immunomodulating cytokines in skin
Effects of ultraviolet and environmental stress
Epidermal stem cells and the hair cycle
Fibroblasts and other cutaneous cell types
Artificial skin substitutes

Skin can be an excellent candidate to study hormesis and its underlying mechanisms because of its accessibility; its repertoire of inflammatory and immunomodulating cytokines, hormones, vitamins, unique responses to ultraviolet light, toxins, and physical injury; and the availability of noninvasive bioengineering and DNA microarray technology. Artificial skin substitutes are also available to study the effects of harmful or dangerous agents. In essence, the skin has everything: from stem cells, signaling, and cellular differentiation, to inflammation, diseases, and cancer. All these facets could become excellent models to further study hormesis and its clinical implications following exposure to a variety of toxic compounds and pharmaceutic agents.

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3 Toward an evidence-based dermatotoxicology

Sebastian Hoffmann, Thomas Hartung, and David Basketter

INTRODUCTION

The term evidence-based toxicology (EBT) was first introduced in 2005. Guzelian et al. (1) used the term in the context of causation and risk assessment in toxicology, whereas Hoffmann and Hartung (2,3) identified commonalities of toxicologic tests assessment and evidence-based evaluation of diagnostic measures in medicine. The link to evidence-based medicine (EBM) was further explored emphasizing the need for more transparent, objective, and consistent approaches in toxicology (4). Especially EBM methodologies, such as systematic reviews, and practices, for example, as established by the Cochrane Collaboration, have been proposed (5,6). However, attempts adopting these approaches to toxicology, for example, as the development of a scoring tool for the inherent quality of toxicologic studies by Schneider et al. (7), remain scarce.

More recently, the concepts of EBT have been put forward (8,9) to help objectively assessing the methods needed and developed for implementing the vision "Toxicity Testing for the 21st Century—A Vision and A Strategy" (10).

Here, it is attempted to review dermatotoxicologic methods from an EBT point of view. The evidence base of currently regulated and state-of-the art test methods for hazard identification of chemicals/cosmetic ingredients to induce skin irritation/corrosion and skin sensitization will be evaluated. The focus is especially set on the aspects of types of evidence, evidence quality and relevance, and evidence synthesis. These criteria will be generally introduced as a precursor to the specific consideration of dermal health effects.

FROM EBM TO EBT

EBM and more generally evidence-based health care (EBHC) have started to develop in the 1970s. Today it has grown to a widely accepted approach to carefully review clinical methods, especially in diagnosis, prognosis, and treatment, on the basis of research evidence, clinical expertise, and individual circumstances to identify the best practice for each patient, taking personal preferences into account (11,12). In essence, this approach requires that the patients and the physician interact on the basis of the relevant research evidence. Ultimately, the synthesized evidence is applied to the patient's health care. EBM first entails framing the question at hand as a guide for the information search. Next, the search is thoroughly planned, ideally the search strategy and analysis procedure peer-reviewed, carried out, and then fully documented. The retrieved information is critically appraised according to predefined criteria distinguishing different evidence levels that range from expert opinion to randomized trials.

A pivotal tool in this process covering the search, appraisal, and evidence synthesis are systematic reviews, for example, as described for laboratory medicine (13). To make the scientific evidence available for the medical community, EBM elaborates systematic reviews and disseminates them via the Cochrane library. In contrast to narrative reviews, which are more prevalent in toxicology, systematic reviews manifest a transparent and objective approach. By a priori specification of search criteria and the data appraisal and synthesis, it becomes a reproducible process. Furthermore, it strives to minimize the influence of potential biases. If appropriate and possible, but not necessarily, meta-analyses are performed to synthesize the appraised evidence by extracting the data from the individual information sources and summarizing these (14,15).

Although the principles of systematic review should be readily applicable to toxicologic questions, such as those regarding hazard and risk assessment of chemicals, attempts in scientific literature are rare and are most often related to occupational health issues (e.g., (16,17)). In particular, the work by Rudén on trichloroethylene and by Golden et al. on Polychlorinated Biphenyls (PCBs) (18,19), which is related to risk assessment of chemicals, highlighted the need for more systematic approaches in toxicology as a basis for more consistent decisions. Furthermore, systematic reviews would serve the recent demand for more transparency (20) and provide means to assess biases in toxicology (21).

Essential to the data appraisal of systematic reviews, is a harmonized scheme to assess the quality of the data. Only a structured and widely accepted assessment scheme allows consistent ranking of data according to their quality. Usually EBM distinguished five levels of evidence ranging from "expert opinion" (level 5) to high-quality studies, such as randomized clinical trials, and systematic reviews thereof. A detailed table presenting the evidence levels for various fields, such as, diagnosis or prognosis, can be found on the website of the "Centre for Evidence Based Medicine (CEBM)" (22). Once having defined the level of evidence, every piece of evidence needs to be evaluated in detail. Basically, for all clinical fields and evidence levels, a variety of appraisal tools exist. Such tools usually provide a list of questions, which are to be answered for each piece of evidence. Examples of such tools in the field of diagnosis are QUADAS and QAREL (23,24).

This need for guided assessment has been recognized in ecotoxicology (25,26) as well as toxicology (7,27,28). However, the proposed methods either fall short of providing an objective tool, especially the method proposed by Klimisch et al. (27), as they do

not require a justification of the assessment, or they have not (yet) reached a broader audience. The work of Schneider et al. (7) places itself specifically in the frame of EBT. By means of two rating experiments assessing a range of toxicologic papers, appraisal tools were developed and improved for in vitro and in vivo methods. Although of potential use as a tool for the assessment of (eco-)toxicologic studies and publications, as for example required according to the new European Chemical Regulation REACH (Registration, Evaluation and Authorisation of Chemicals) when submitting data, it seems that it has not been used to any major extent. This is unfortunate, including for dermatotoxicologic methods, as a transparent and comparable quality assessment of data would have been extremely beneficial for the exploration of the vast amount of toxicologic test data generated under REACH, which is made to some extent accessible from the European Chemicals Agency (ECHA).

Furthermore, EBM has a lot to offer for toxicologic test method assessment. Especially in the field of evidence-based evaluation of diagnostic tests, approaches and methods have been and are developed that also offer a lot of potential for toxicology (2). Crucial aspects necessary for understanding a toxicologic test method are very rarely considered, so that in most cases sensitivity, that describes a test's ability of a test to detect positives in a population of positives, and specificity, that describes a test's ability to detect negatives in a population of negatives, are used as the parameters driving the tests assessment. This holds true also for dermatotoxicologic tests, for example, for skin corrosion (29) and skin irritation (30), not at least because of formal requirements, as for example, defined in the "Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment" (31). This approach neglects to a large extent that sensitivity and specificity are mutually dependent. Their balance can be adjusted by moving the threshold that is used to discriminate the negatives from the positives. Therefore, receiver–operation curves, which allow this to be taken into account, are a standard tool in EBM diagnostics (32). In addition, sensitivity and specificity describe a test's performance in a given experimental setting, which is often designed to produce reliable estimates of the two. This potentially results in a very artificial situation, that is, a balanced design of the same number of positive and negative reference test samples, which is equivalent to a prevalence of 50% positives. Subsequently, it is still required to plug the information obtained (sensitivity/specificity) into a real-life situation with possibly very different prevalences. To achieve this, other parameters, such as odd ratios or predictive values, are helpful.

Inherent not only to sensitivity or specificity, but also other parameters used to assess test methods, is the problem that the reference standard is not perfect. To select a number of negatives and positives, definition criteria are needed. Quite often in toxicology, an established test is used to do this. It is well recognized that this established test is also only a model for the true human toxicity and hence not perfect. But in the absence of obvious solutions accounting for this imperfection in the reference standard, the established test is (inappropriately) considered as perfect. However, diagnostic test assessment has developed several approaches to remedy this situation (33), some of which have already been proposed for use in toxicology (34). Especially in this aspect, the movement driving the whole of toxicology to an understanding of the pathways of toxicity, for example, as an, if not the essential concept of the vision of the toxicology of the future (10,35), and the concepts of an EBT intertwine. One the one hand, the need of pathway toxicology to minimize or, if possible, eliminate the species differences resulting from the extrapolation from animal model data to humans will reduce the issue of imperfectness of references in test assessment. On the other hand, pathway toxicology will generate vast quantities of evidence, which will need to be reviewed systematically, appraised and synthesized, while the resulting methods will need to be developed according to quality standards followed by a thorough assessment (9). Furthermore, the integration of the information will require computational tools, which although complex, usually are consistent and can be made transparent. This contrasts the current practice of weight of evidence (WoE) approaches in toxicologic decision making, which are often subjective and not transparent and thus difficult or impossible to reproduce. The concept of WoE and its shortcomings have been explored in detail by Weed (36).

Of the here described aspects of EBT, especially the topic of test assessment is of special interest for dermatotoxicology, as it offers several advantages for pioneering transparent, consistent, evidence-based approaches in toxicology.

SKIN IRRITATION

With emerging political pressure in the 1990s to develop in vitro methods to replace the respective in vivo methods, especially in Europe, research in the field of skin irritation focused on the development and validation/assessment of in vitro methods for hazard identification (37). In order to do so an understanding of the biological effects to be mimicked in vitro is required. The mechanism of irritation is, however, not yet fully explored (38,39), not at least research has been attracted to the more severe human health effect of skin sensitization.

Nowadays, hazard identification for irritation is almost exclusively performed in vitro. The respective tests have undergone a long process of development, assessment, and review, which first of all was characterized by sustained scrutiny. As a consequence, a lot of the above-described aspects of EBT have been applied, although not explicitly and consistently.

Skin irritation opened, and still continues to open up, several avenues to explore in detail test assessment aspects of EBT. Indeed, it has been used as a test case to introduce more evidence-based approaches, for example, with regard to the definition of a reference standard or in the context of a more comprehensive assessment of test performances (40). One pivotal reason for this is the availability of good quality in vivo data, both rabbit, as used in the standard animal model, and humans.

Assessment of Reference Tests for Skin Irritation

Traditionally, the skin irritation potential of chemicals has been assessed with the Draize rabbit test for skin irritation using a subjective scoring of effects, mainly erythema, edema, and persistence, usually 24, 48, and 72 hours after a 4 hour application (41,42). Performance of this Draize test has been evaluated in some detail, although not in a systematic manner, mainly in the context of assessing and introducing the respective in vitro epidermis models. If run under controlled conditions, such as Good Laboratory Practice (GLP), the reproducibility of the Draize test for skin irritation seems to be acceptable (43), while it has been shown that it is oversensitive when compared with human patch test data (44,45).

However, the rabbit test will increasingly be replaced by the in vitro test based on reconstituted human epidermis/skin, for example, as currently taking place under the REACH regulation. Consequently, future test methods will refer at least to some extent to data from in vitro tests. Although these in vitro tests have been thoroughly evaluated/validated before their regulatory acceptance, regular use will steadily increase the evidence base of their performance. Systematic collection and review of this evidence as it has been identified as a potential aspect of EBT, will not only help to improve the understanding of capabilities and limitations, but will provide a sound basis for future developments (46). However, mechanistic and "-omics" research to advance the mechanistic understanding of skin irritation and ultimately delivering more predictive biomarkers and innovative testing opportunities remain scarce (39,47,48).

Assessment of New Tests and Test Strategies for Skin Irritation

In toxicologic hazard assessment, new tests are usually assessed by comparing the test results with predefined reference results for a defined set of chemicals (34)—an approach methodologically similar to the standard evidence-based solutions in clinical diagnosis (49). In case the reference results are defined by a single reference test, a prospective study on a common set of chemicals running the reference test and the new test in parallel would represent a favorable experimental design. However, such an approach is resource intensive and is ethically not always feasible if the reference test uses animals. Therefore, reference results are usually established by exploiting existing data. This was, for example, the case in the assessment of the human epidermis models for skin irritation. In the evaluation of these models the Draize test for skin irritation was chosen as the reference test, while the respective reference results were derived from existing Draize test data compiled from several databases of sufficient quality (50). As the Draize test is a far from perfect reference, such a direct comparison produces the challenge to take into account its imperfection. Simply assuming that a reference test is a perfect reference introduces bias, which renders a systematic and objective test assessment almost impossible.

While the incorporation of reference tests' imperfection is one methodologic aspect of EBT, the increasing availability of information and relevant data that render test assessment more complex is another that demands new conceptual approaches. Only a systematic approach considering all potentially informative evidence will allow a consistent and comprehensive assessment of tests intended to inform skin irritation hazard for humans. Here, dermatotoxicology in general, but especially skin irritation, can be considered as predestined to play a pivotal role in exploring EBT approaches and methodologies.

Various types of information and data may contribute to the assessment of a chemical's potential to induce human skin irritation. Figure 3.1 provides, without claiming completeness, a general framework summarizing such potentially relevant evidence. The four information categories of physicochemical, human, in vitro, and in vivo data are distinguished. Each category comprises several subcategories. Correlative approaches such as read-across and (quantitative) structure–activity relationships [Q(SARs)] have not been considered, as these are not directly determined. Furthermore, their performances still need to be improved (40). Nevertheless, the chemical structure as listed under the physicochemical data might be informative when studying mechanistic issues. Obviously, some subcategories will provide much more information than others. For example, human patch test data will be considered of higher human relevance and thus of greater value than data from in vivo or in vitro tests. In addition, the available evidence will differ from chemical to chemical leading to varying degrees of (un-)certainty. These aspects considerably increase the difficulty when integrating/synthesizing the evidence to compose a reference for comparison.

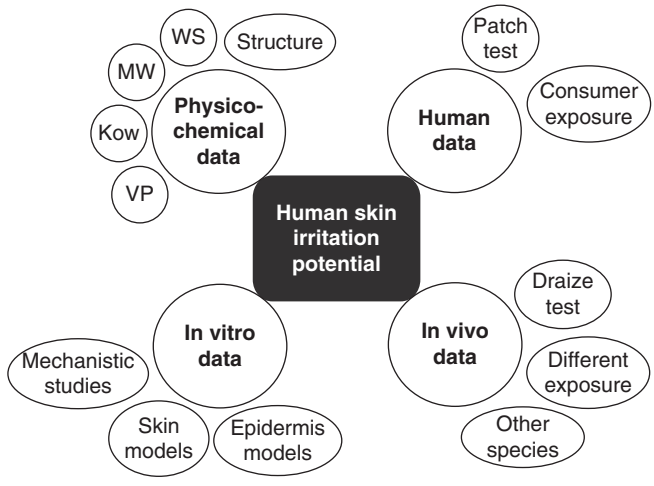

FIGURE 3.1 Data informing human skin irritation potential. *Abbreviations*: MW, molecular weight; WS, water solubility; VP, vapor pressure; Kow, octanol–water partition coefficient.

In toxicology almost unique, however, is the possibility to obtain human information. Standard protocols for human patch tests exist (44), so that results from these tests can be considered as an (almost) perfect reference standard. This allows to a large extent circumvention of the problem of imperfection in the assessment of new tests. In addition, it offers a means to transparently and consistently explore how to best synthesize evidence (40), ultimately providing a unique database allowing the development and evaluation of newly developed evidence-based methods and methodology for toxicologic test assessment. More details on this EBT aspect of systematic, transparent, and consistent data integration/synthesis are presented in the following chapter on skin sensitization assessment.

SKIN SENSITIZATION

Traditionally the human skin sensitization potential of chemicals has been assessed by a heavy reliance on animal models. The first regulated test using guinea pigs (51,52) have to a large extent been replaced by the murine local lymph node assay (LLNA) (53,54) and more recently its nonradioactive variants (55).

Due to deeper mechanistic understanding of the process leading to skin sensitization in humans combined with political pressure in Europe demanding animal-free evaluation of skin sensitization of cosmetic ingredients, several in vitro methods are emerging (56). Some of them especially related to the earlier events in the process of skin sensitization, such as protein reactivity and activation of dendritic cells, have reached a level of standardization qualifying them for formal assessment (57–60).

In parallel, the political demand has led to considerable efforts in framing the task and providing regular records of progress (61–63). Indeed, this frequent review, although not (yet) systematically, serves as a role model of a continuous adaption to advances in the field as required for a steadily updated evidence-based test assessment.

Similar to skin irritation, the field of skin sensitization can be regarded as an opportunity to advance evidence-based approaches in toxicology. The growing mechanistic insight and the increasing database offer the unique opportunity to explore ways to synthesize evidence in transparent and consistent manners.

Test Assessment in Skin Sensitization

Historically, guinea pig tests, notably the Buehler Test and the Guinea Pig Maximization Test have been the cornerstone of hazard identification for skin sensitization. These methods recapitulated the entirety of the sensitization induction and elicitation process, measuring the extent of any response largely by changes in erythema at the skin test site. In more recent years, a refined and reduced assay, the LLNA has taken precedence, being a test with an objective, quantitative endpoint—cell proliferation in draining lymph nodes. The LLNA was the first alternative to undergo formal validation and, remarkably, this assessment took into account reference results from the previous guinea pig-based tests as well as human data (64). This approach with two points of reference provides the basis for the construction of a composite reference standard. As a consequence of this, it can be reasonably assumed that upcoming in vitro tests—either as stand-alone or in a strategic combination—will be compared against a reference composed of guinea pig, human, and LLNA data, as displayed on the right of Figure 3.2. However, so far the data have primarily been interpreted separately, that is, by comparing LLNA data versus guinea pig and versus human data. Integration of both was done to some extent in an expert-based WoE approach, but no attempts have been made to synthesize the evidence in a structured and transparent way. It is worth noting that this may be particularly important in relation to human data, which may be derived from experimental studies, such as the human repeated insult patch test as well as from clinical investigations. This latter source often involves ad hoc diagnostic investigations reported as individual or small groups of clinical cases. Both the quality and reliability of data from these need vigorous justification.

Furthermore, the mechanistic understanding, as for example, briefly outlined by Aeby et al. (65) and the way this is reflected in the in vitro tests adds another dimension of complexity to test assessment. While it strongly contributes to the evidence basis in the assessment, especially the importance of the different events in relation to each other in the process of human skin sensitization poses new challenges for evidence synthesis.

At some point it will be necessary to integrate consistently the available evidence in a transparent way to provide a point of reference to support the development of testing strategies. Furthermore, this will help to quantify the contribution of the core events in the process of skin sensitization, such as bioavailability, skin metabolism, protein reactivity, maturation of dendritic cells and proliferation of T cells, and their dependences.

Construction of Test Strategies for Skin Sensitization

The challenge of performing human skin sensitization hazard and risk assessment without animal models has resulted in remarkable advances in the field. It has been recognized already when developing the LLNA that the "blackbox" guinea pig models need to be replaced with test methods reflecting the biological processes of human skin sensitization. Basic research has provided the insight that allowed mapping the key events in that process. These events, in their biological sequence, are used as the building blocks that need to be covered to master the challenge. Consequently, in vitro and in silico approaches modeling the specific events, as shown on the left of Figure 3.2, are being developed.

The initial event is the bioavailability of a compound. It determines if further assessment is required. If bioavailability is negligible, the hazard and risk assessment can be concluded. Bioavailability is directly linked to physicochemical properties, such as the molecular weight or the octanol–water partition coefficient. Furthermore, it depends on exposure determinants, such as duration and concentration. As experimental approaches, for example, measuring skin penetration, have limitations, not only the analytical challenges, but also in understanding concentration and

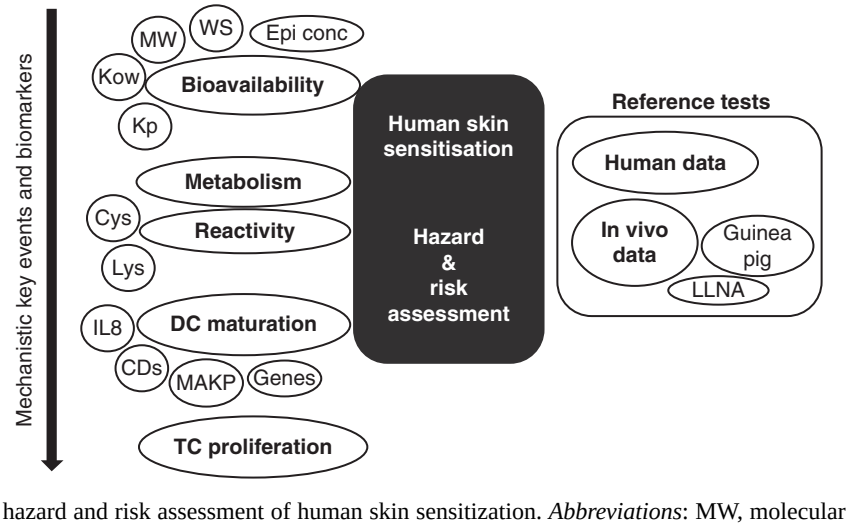

FIGURE 3.2 Data informing hazard and risk assessment of human skin sensitization. *Abbreviations*: MW, molecular weight; WS, water solubility; Kp, permeation coefficient; Kow, octanol–water partition coefficient; Epi conc, epidermal concentration; Cys, Cysteine; Lys, Lysine; IL8, Interleukin 8; CDs, proteins, for example, CD86 and CD54; MAKP, p38 MAP kinase; DC, dendritic(-like) cells; TC, T cells.

duration of sensitizer at the real target site(s) in skin, generally in silico or toxicokinetic models using physicochemical information are used to inform bioavailability (66). In only a few cases, however, such models allow reliably the exclusion of bioavailability.

Once in the epidermis, the metabolic or oxidative processes either activating or inactivating a compound in terms of its reactivity need to be considered. While in silico models may inform this event (67), it would be desirable to include this aspect in in vitro methods, which primarily have been developed to model the subsequent key events. Indeed, metabolic-competent variants of in vitro tests addressing a compound's reactivity, that is, the next key event, are being developed (68). In most cases, at least some reaction with skin proteins is required to induce maturation of dendritic cells. The event of maturation is being modeled in vitro using dendritic or dendritic-like cell lines measuring different biomarkers (59,60,69). The final event is T-cell proliferation. As reviewed by Maxwell et al., more efforts are required as no promising in vitro models are available yet (63).

In summary, for each key event in skin sensitization, in vitro and in silico approaches are available or under active development. This presents the challenge to combine them to construct an integrative testing strategy (ITS) (70,71). It has been realized that decision-tree-like flowcharts relying to some extent on expert WoE will neither allow to appropriately address the complexity of the underlying data, nor to identify optimal testing sequence (72). Therefore, a framework and methodologies have been proposed that match with the concepts of EBT (72,73). The ITS-framework is in essence defined by two basic characteristics: it should result in consistent decisions and it should be transparent, so that it can be adjusted when new evidence becomes available. In addition, it should also be efficient in the sense that it balances the testing costs and testing time against the reduction of uncertainties or the likelihood of making wrong decisions. For some purposes, for example, when assessing the skin sensitization risk for low exposures, less evidence—being usually associated with higher uncertainty—might be sufficient.

In this context, Bayesian networks (BNs) have been proposed as a methodologic approach that can fulfill the requirements of the framework. Although they are case-/chemical-specific, BN also allow assessing and incorporating conditional dependences of tests. In the case of skin sensitization, dependencies will primarily exists along the biological sequence of the key events. For example, high protein-reactivity may likely imply increase maturation of dendritic(-like) and T-cells. As a consequence, BNs even are capable of guiding testing, for example, by proposing to stop testing in cases when no added value of information can be expected.

Remarkably, Jaworska and Hoffmann when further developing the framework and discussing methodologic requirements chose skin sensitization as the human health effects to initially explore and demonstrate the suitability of BN (73). This initial activity has been developed in more detail resulting in a proof of concept showing that no generic optimal testing strategy exists, but that they depend on the available case-specific evidence (67).

This incorporation of the mechanistic understanding of the skin sensitization process together with novel approaches to synthesize evidence in an ITS-framework is an outstanding early example for a transition to an EBT. Although not always placed in the EBT-frame, it is pioneering the path from animal models to mechanistically based, systematically evaluated and transparently and consistently integrated in vitro models.

In addition to the above for skin sensitization, it is important to mention that the main in vivo method defines not only hazard, but also the potency of that hazard. It is not relevant to review that topic in detail here, but it does provide a key opportunity to put into practice the principles of EBT. The LLNA estimate of relative potency has made a major contribution to skin sensitization risk assessment, but it does not represent the "gold standard," such does not yet, if ever, exist (62,74). Thus, we have the chance to apply EBT principles to the use of in vivo and human data to the establishment of a coherent set of substances whose relative potency is fully characterized and which will provide the true standard against which in vitro determinations of potency can be assessed.

CONCLUSION

The concepts of an EBT have been adopted and adapted from the field of EBM and health care (4). The essence of EBT constitutes the demand for systematic, transparent, and consistent approaches in toxicology. Critical views of EBT claiming that practices always have been based on evidence are not convincing. In the light of heavy reliance on animal models that have to a large extent never been systematically evaluated, narrative and thus subjective review practices and expert-based weight of evidence, a transition to the principles of EBT is highly warranted. Such a transition will advance toxicology facilitating the full exploitation of new technologies and the ever increasing wealth of information. In addition, EBT is anticipated to serve as a quality assurance tool when implementing the vision of a toxicology for the 21st century (8,9).

So far, only limited work has been carried out to explore and apply the tools proposed under EBT. Some systematic reviews addressing individual substances and the available human and animal data are available (75), but systematic reviews of test methods as undertaken in clinical diagnostics are still lacking. Few approaches to systematically assess the quality of (eco-)toxicologic evidence have been proposed (7,25,26). Similarly, some ground work on fundamental problems of test assessment has been presented (2,40). In contrast, the EBT aspect of evidence synthesis has attracted considerable attention. Fuelled by European policy, novel approaches to integrate evidence from a variety of information sources are being developed. Building in mechanistic knowledge, the fundamental properties of the frameworks largely converge with those of an EBT (73). However, the problems faced have different facets as compared with medicine. The very different and heterogenous information that may be relevant represents a variable and complex evidence base that cannot be handled simply by adapting EBM methodology. EBM tools need to be expanded and combined with sophisticated methodologies of evidence synthesis.

When reviewing EBT, it is remarkable that in many instances dermatotoxicologic health effects, especially skin irritation and skin sensitization, have served, either explicitly or unintentionally, as the "guinea pig" to explore EBT concepts and tools. The availability of good quality data from rabbit Draize tests for skin irritation and highly relevant human patch test facilitated the conduct of more informative test assessments of the epidermal skin irritation models for hazard characterization (40). Similarly, also in the field of skin sensitization the need for more comprehensive test assessment has been recognized. New tests are compared with several reference standards, such as guinea pig models, LLNA, and human

data. However, evidence-based approaches that enable a single, comprehensive comparison, such as constructing a composite reference standard, have not yet been explored. In addition to their primary purpose of improving evaluation of new tests for the respective human health effects, these approaches toward a more evidence-based test assessment explore and advance generic tools of EBT.

Furthermore, the push for animal-free hazard and risk assessment of chemical skin sensitization fosters the principles of EBT. It has been recognized that this can only be achieved by integrating evidence from a variety of sources that are mapped onto the frame of up-to-date mechanistic understanding (61). To provide a sound basis for further advances in the field, improved knowledge or introduction of new tests, the evidence integration/synthesize has to fulfill several requirements. These requirements of transparency, consistency, and rationality are identical to those of EBT. As a consequence, while searching for solutions to appropriately assess skin sensitization, new methodologies are being developed and explored that are evidence based by definition. This adds considerably to shaping EBT and fills its generic tool box.

It can be anticipated that the lessons learned and advances achieved in the field of skin sensitization will be of crucial importance when the even more complex human health effects will be addressed. Especially in view the pathway/mechanism-driven vision of a toxicology for our century, EBT may provide essential methodologic approaches. Systematic reviews would allow an evidence-based evaluation of pathways and their mapping (76). The resulting (in vitro) tests would be made comparable if assessed with appropriate and comprehensive evidence-based tools (9). Similarly, evidence synthesis methodologies that allow accounting in detail for pathway/mechanism knowledge and are compliant with the core principles of EBT can provide the urgently needed 21st century tools to help implementing the vision of a new toxicology.

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4 How to improve skin notation

Pietro Sartorelli, Heinz W. Ahlers, and Jesper B. Nielsen

INTRODUCTION

In many countries compounds considered to be a skin hazard are identified by a skin notation (*S*) on the list of occupational exposure limits (OEL). The *S* was introduced about 50 years ago to alert attention to the fact that dermal exposure to these compounds can significantly contribute to the total systemic exposure. Thus, irritating and corrosive compounds were originally not intended to have *S*. Today up to one-third of all industrial chemicals in the OEL lists of many countries have the *S*. However, a general agreement on the *S* assignment criteria does not exist even when the different countries have close to identical OEL for inhalation exposures. In the past many inconsistencies in the assignment of *S* from both American Conference of Governmental Industrial Hygienists (ACGIH) and national lists were found (1–3). Nielsen and Grandjean (4) compared the use of *S* on OEL lists of Denmark, Germany, Netherlands, Poland, Slovakia, and US ACGIH, and demonstrated substantial differences between countries that otherwise have very comparable OEL. Similar proportions of chemicals with *S* were present in the OEL lists in the six countries (Table 4.1), but in many cases *S* was assigned to different chemicals (Fig. 4.1).

Generally *S* should be assigned for substances with a low dermal LD_{50} (less than 2000 mg/kg). However, the present use of *S* does not reflect this proposal (Table 4.2) as only few countries assigned the *S* to all such chemicals on their OEL lists (4). ACGIH's criteria were never clear, so that for many of the compounds with the *S* the documentation did not refer to published data (2,3). These inconsistencies could be attributed to different factors, namely, lack of systematic dermal absorption studies on chemicals of occupational interest, conflicting information on dermal absorption obtained with different experimental systems, dependence of the absorption rate on exposure conditions, and lack of criteria to define the importance of skin penetration in occupational exposure to chemicals. Additionally, the lack of a warning for substances that were extremely corrosive or irritating to the skin itself sometimes resulted in *S* being inappropriately assigned to these hazards. Moreover, *S* represents a rigid criterion to distinguish between compounds that can definitely cause systemic effects due to limited skin contact and nonhazardous compounds. In other words, *S* is a qualitative hazard indicator while workplace exposures should be evaluated in quantitative terms. This concept seems very limiting as the cases of acute intoxication are relatively unusual today. Furthermore, *S* does not take into account a number of situations in which most of the workplaces are involved, such as dermal exposure to mixtures, increased percutaneous penetration in certain dermatologic diseases and skin contamination with percutaneous penetration enhancers. To this we must add the lack of consensus on quantitative dermal risk assessment that at the moment prevents the use of dermal occupational exposure limits (DOEL) mainly due to (5):

- lack of validated and standardized techniques of dermal exposure measurements (there is no general agreement on how to measure skin contamination);
- difficulty in evaluating the extent of contaminated skin;
- regional variations in skin permeability;
- lack of percutaneous penetration data;
- influence of worker's behavior on skin contamination.

At the 28th International Congress on Occupational Health (Milan, Italy, 2006) a Satellite Workshop on "Dermal Risk Assessment at Workplace" was organized by the Scientific Committee on Occupational and Environmental Dermatoses of the International Commission on Occupational Health (ICOH SC OED) with the aim of exploring the actions needed to improve the *S* system in an international harmonized perspective. A position paper was published focusing the following 'aspects (6):

- At the time *S* was the only example of a regulatory tool for dermal risk communication. Various international agencies and individual author groups proposed new strategies for development of *S* that differed from each other in a number of ways, but generally suggesting that *S* should be based on human/animal evidence when available, or otherwise supported by experimental evidence based on internationally accepted methods [e.g., derived following existing guidelines of the Organization for Economic Co-operation and Development (OECD)] or on mathematical/probabilistic models, such as [quantitative] structure–activity relationships ([Q]SARs). Unfortunately, human in vivo evidence (i.e., credible evidence indicating the incidence of systemic effects among workers as a result of chemical exposure) is difficult to obtain and decisions are mostly likely to be based on in vitro experimental evidence, models, or animal data that relate the absorbed dermal dose to the calculated dose from inhalation.
- Different vehicles and co-exposure to detergents or water will significantly change dermal absorption.
- There are no specific guidelines for preventive measures linked to the *S* other than vague recommendations, such as "prevent skin contact" and there is a lack of information on effectiveness of protective measures. There is evidence that in many cases *S* is neither understood nor widely used by industry, and in this sense it is not useful in the control of dermal risks.
- A grading of *S* based on toxicologic and percutaneous penetration data may increase the usefulness of the notation.

TABLE 4.1
Number of Chemicals on National OEL Lists and Chemicals with Skin Notation

	Chemicals in OEL Lists	Chemicals with Skin Notation	
Countries	**n**	**n**	**%**
Germany	684	176	26
USA	650	192	30
Denmark	634	204	32
The Netherlands	676	157	23
Poland	414	144	35
Slovakia	269	85	32

Abbreviation: OEL, occupational exposure limit.
Source: From Ref. 4.

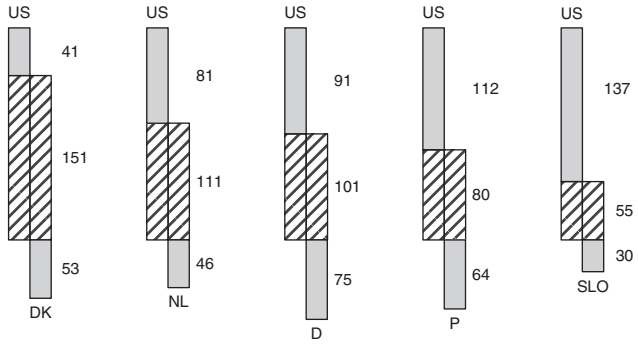

FIGURE 4.1 The number of chemicals with a skin notation in the United States as compared with five other countries. Hatched overlap indicates the number of chemicals given a skin notation in both countries. US, USA; DK, Denmark; NL, Netherlands; D, Germany; P, Poland; SLO, Slovakia. *Source*: From Ref. 4.

TABLE 4.2
Presence or Absence of Skin Notation for Five Chemicals with a Dermal LD_{50} Below 2000 mg/kg

Chemicals	Denmark	USA	The Netherlands	Germany	Poland	Slovakia
Ethylamine	+	+	–	–	+	–
Cyanamide	–	–	–	–	+	–
Methacrylic acid	–	+	–	–	–	–
Sodium azide	–	–	–	–	–	+
Acroleine	–	+	–	–	–	–

Source: From Ref. 4.

- *S* must remain a hazard indicator to be used by professional occupational hygienists and should not be considered as an alternative to dermal risk assessment.
- It might be possible to use percutaneous flux in place of *S*, although some (semi)quantitative indicator of uptake flux would be needed.
- Biological monitoring (whenever possible) is necessary for chemicals with *S*.

The Skin Notation Workshop organized by ICOH SC OED hosted in the 11th International Percutaneous Penetration Perspectives Conference (La Grande Motte, France, 2008) represented the continuation of this activity (7). Participants considered that in the future, with the introduction of the EU REACH (European Union Registration Evaluation Authorisation and Restriction of Chemicals) regulation, manufacturers have to provide information on dermal uptake of their products and this will allow regulatory bodies to obtain more information on this route of entry for pure substances as well as for mixtures. In their opinion *S* could be improved by using available experimental data and including information on systemic toxicity, sensitization, irritation, and carcinogenic effects. In this way perceptions and understanding of skin hazards will increase. Some of them thought that *S* could be a semi-quantitative hazard indicator and the risk for systemic toxicity or skin damage could be rated, for example, by using a traffic light system. Better perceptions and understanding of *S* will help in preventing contact, and local and systemic effects with the aim of improving safety at workplace.

Today, besides ACGIH, the points of reference in the specific field are the SCOEL (Scientific Committee on Occupational Exposure Limits) and the German *S* systems, while a new approach to *S* has been proposed by US National Institute for Occupational Safety and Health (NIOSH).

THE SCOEL APPROACH TO SKIN NOTATION

SCOEL developed a guidance note in which one of the chapters addresses *S* (8). According to the guidance note, the *S* assigned to an OEL warns of a possible significant contribution of dermal absorption to the total body burden (6). The interpretation of "significant" is established on a case-by-case basis, but may in general be of the order of 10% or more of the respiratory uptake during exposure at the OEL. The criteria proposed by SCOEL were developed by the Dutch Expert Committee on Occupational Standards (DECOS) and has also been proposed by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC). The ECETOC proposal is more detailed in that it suggests an *S* when the amount of chemical absorbed upon exposure of both hands and lower arms (2000 cm^2) for 1 hour is expected to contribute more than 10% to the systemic dose, compared with the amount absorbed via inhalation exposure at the OEL during a full workday, assuming that 10 m^3 air is inhaled during an 8 hours workday and that 50% is absorbed. However, this definition will apply only for chemicals where the OEL is based on systemic toxicity (9).

The *S* does not relate to, and is not intended to give warning of direct effects on the skin, such as corrosion, irritation, and sensitization. SCOEL lists the following factors as determinants of the extent of dermal absorption (7):

- amount of substance in direct contact with skin (i.e., dose);
- physicochemical properties of the substance;
- co-exposure to vehicle or other chemicals that may enhance the penetration;
- duration of exposure;
- physical form of the substance.

SCOEL further discusses volatility. Substances with high boiling temperature and low vapor pressure may give rise to skin exposure not only directly from contaminated air but also indirectly via deposition of aerosols. On the other hand, substances with high vapor pressure are likely to evaporate rapidly, thus decreasing the potential for dermal uptake.

As possible quantitative data sources the following are mentioned:

- direct dermal uptake measurements (in vivo/in vitro, human/animal);
- comparison of dermal LD_{50} with intravenous or intraperitoneal LD_{50} values.

Furthermore, evidence of significant dermal uptake may be obtained from

- case reports of systemic effects following skin exposure;
- substantial variation in biomonitoring data in groups with similar air exposure levels;
- phenomena such as subjective taste after "skin only" exposure.

In the absence of other data, an indication of likely skin penetration may be inferred from physicochemical data and/or [Q]SARs.

Biological monitoring may reflect systemic exposure (and health risk) better than air monitoring for substances that are absorbed through the skin. Consequently, SCOEL decided in 2005 to always try to set a biological limit value for compounds with the *S*.

ASSIGNMENT OF SKIN NOTATION FOR CHEMICALS WITH GERMAN MAK VALUES

In Germany scientifically based occupational threshold values in air (MAK) and in biological materials (BAT) are evaluated by the DFG Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area. In general these values are adopted by the German government and obtain legal status (10). A substance is labeled as absorbable through the skin when the skin contamination endangers the person exposed to it and if adherence to the stipulated MAK value alone no longer gives sufficient protection against damage to health.

A substance is classified as absorbable by the skin if one of the following criteria is fulfilled:

I. Classification on the Basis of Investigations in Man

Field studies or scientifically based case studies show that percutaneous absorption after contact with the substance in question is of practical relevance.

Percutaneous absorption is without doubt responsible for part of the internal exposure and this exposure can lead to systemic toxic effects.

II. Classification on the Basis of Investigations in Animals

Animal experiments show percutaneous absorption and this exposure can lead to systemic toxic effects.

III. Classification on the Basis of In Vitro Investigations

Relevant percutaneous absorption is quantified using recognized methods. The flux through the skin is determined and the permeability constant (Kp) is calculated or will be calculated, or there is data available on the percentage absorption of the applied dose (% absorbed per unit of time and surface area).

IV. Classification on the Basis of Theoretical Models

Relevant percutaneous absorption can be assumed on the basis of analogy or mathematical model calculations.

Criteria I–IV are arranged in order of importance with the data from man being the most important (10). About one-third of all substances are classified as absorbable through the skin according to these criteria.

THE US NIOSH STRATEGY FOR ASSESSING SKIN NOTATIONS

The US NIOSH has adopted a multiple notation strategy for dermal hazards that includes a weight-of-evidence approach utilizing the reports of human exposures and health effects, the empirical data from in vivo and in vitro laboratory testing, the considerations provided by predictive algorithms, such as [Q]SARs, and mathematical models that describe a selected process (e.g., skin permeation) using analytical or numerical methods (11).

The NIOSH notations appear in the NIOSH Pocket Guide to Chemical Hazards beginning in 1990 and had previously been adopted from the US Occupational Safety and Health Administration (OSHA) *S* appearing in the Code of Federal Regulations, 29 CFR 1910.1000, table Z. OSHA in turn had adopted these *S* from the ACGIH 1972 Threshold Limit Value tables. These *S* were subject to the same limitations noted earlier.

In the process of developing this new strategy, NIOSH reviewed the existing NIOSH *S* for 138 individual substances and four substance groups, and noted three major problems:

- the notations in theory are established based on the potential contribution of a chemical substance to systemic toxicity as a result of dermal absorption (54 Fed. Reg. 2718, 1989); however, the assignments of the *S* have not consistently followed this principle and many notations are based only on the potential or reported transdermal penetration of chemicals without considering the link between dermal absorption and overall toxicity;
- the provision of a single *S* limited to systemic toxicity often results in the appropriation of that warning for other serious dermal effects, such as corrosion and sensitization;
- the *S* adopted following the Permissible Exposure Limit (PEL) update project did not include skin exposure precautions made in NIOSH Criteria Documents.

Thus, some NIOSH Criteria Document recommendations for dermal exposure hazards and precautions were not consistent with the *S* in the NIOSH Pocket Guide.

An improved system of *S*, which is needed to standardize the application of the warning and provides for warnings when the skin itself is the target organ, would be as follows.

SK Designates a Dermal Hazard Combined with One or More of the Following Hazard Categories

- SYS: Hazard of systemic toxicity due to dermal absorption.
  - (Fatal) Highly or extremely toxic.
- DIR: Hazard of direct effect(s) on the skin, including corrosion, primary irritation, and reduction/disruption of the dermal barrier integrity.
  - (Irr) a subnotation of DIR indicating a skin irritant.
  - (Cor) a subnotation of DIR indicating a skin corrosive.
- SK–SEN: Hazard of allergic contact dermatitis, or sensitization of skin, mucous membranes, or airways due to dermal exposure. This sensitization category would be similar to the R42/43 and R42 risk phrases used in the EU system.

The following categories indicate a lack of dermal hazard, a lack of sufficient data or that an evaluation has not been completed.

- SK: The reviewed data identified no health hazard associated with dermal exposure and did not support the assignment of one of the dermal exposure categories.
- $ID^{(SK)}$: There are insufficient data to determine the dermal hazard.
- ND: The substance has not been evaluated under the improved skin notation and dermal hazards are unknown.

The utilization of multiple notations to designate the major adverse health effects from skin exposure allows a distinction between the nonsystemic effects due to skin exposure and the systemic toxicity from dermal absorption, and provides clear warnings to workers. Another improvement is that a combined assignment will accommodate the presence of multiple skin hazards by stringing the hazard categories behind the SK notation (e.g., SK–SYS–DIR). As the scientific data, test methods, and understanding of toxicologic mechanisms involved in skin injuries improve, additional categories may be added to the *S* infrastructure and the current criteria may be revised to enhance the clarity of the notations for the selection of exposure prevention strategies. The proposals for evaluating and assigning *S* are based on the experience gained in developing the NIOSH proposal and current approaches from other standards setting bodies or presented at the Occupational and Environmental Exposures of Skin to Chemicals Conferences (OEESC) in Washington, DC, 2002, and Stockholm, Sweden, 2004. The schematic representation of the US NIOSH strategy of assessing *S* is reported in Figure 4.2.

NIOSH intends to publish a specific profile for each *S* developed under the new strategy. The NIOSH *S* profiles are intended to document the data evaluated in the assignment of the *S* and include information that may be useful to the industrial hygiene professional in assessing the severity of the risk in the workplace. NIOSH has completed and published the *S* profiles for 20 substances. The NIOSH approach is described in detail (11) and these profiles are available electronically at http://www.cdc.gov/niosh/docs/2011–136/ to http://www.cdc.gov/niosh/docs/2011–155/ (12–31).

PERSPECTIVES ON THE SKIN NOTATION SYSTEM

Thorough reviews on the use of *S* have revealed that in many countries in Europe and in USA, the existing systems are based on unclear definitions or an assignment policy that deviates from stated guidelines, for example, assigning the *S* to chemicals with only irritative or corrosive hazards. Along with this, more knowledge on chemicals not previously known to be hazardous to the skin (e.g., sensitizers, irritants) and on the ability of chemicals to be absorbed and cause systemic toxicity has become available. These developments have caused the number of chemicals with *S* to increase. Furthermore, development and increased use of methods based on structure–activity relationships (SARs) to identify hazardous chemicals has added more *S* to the OEL lists, although the latter approach has recently been questioned as no uniformly accepted method apparently exists for such SAR calculations (32).

The present situation is that one-third of all chemicals are given the *S* and that workers are generally not able to discriminate whether the *S* is given because of an irritative potential, a risk for developing of allergy, a corrosive risk, or a chemical that could potentially cause a fatal systemic intoxication. Also workers often assume that the absence of the *S* means dermal protection is not required. This situation is neither acceptable from a professional nor from a preventive perspective.

With one-third of chemicals having the *S*, the warning loses its value, or it is turned into something very general advising the user to avoid skin contact with chemicals. A potential risk is that workers may get used to work with some specific chemicals with the *S* in their work place and experience they, despite skin contact, do not suffer any toxicity, either because the exposure is too low to cause toxicity or because they do not easily become sensitized. This way the workers may lose their respect for the warning and when meeting a new chemical they only notice that it has the same *S* mark as they are used to, and therefore do not expect any toxicity, while this new chemical may potentially cause fatal intoxication. Thus, the workers become ignorant to the warning because most chemicals have the *S* sign and respond to the warning with a "so what."

The recently published NIOSH approach is a step forward because it accommodates information on different effects in relation to dermal exposure within the same marking (e.g., SK–SYS–SEN). Although the same information is present in the EU system through the R-sentences, the information is split and requires the user to know and remember the different risk phrases. However, the NIOSH approach still does not answer the need for a differentiation with respect to severity of risk even if the 20 chemicals for which NIOSH has published *S* profiles do contain information that could be related to the severity of the risk (e.g., where available the penetration data is included in the profile). Actually it is not clear if a SYS warning is more important/hazardous than a SEN warning. After an extensive skin contamination (i.e., an arm) with parathion a worker may die, while if he does the same with another chemical he could run the risk to develop allergy in 10 years. Furthermore, many chemicals with a SYS warning may have toxicities that differ by orders of magnitude. The NIOSH approach does not help here, and it does not consider mixtures and penetration enhancers.

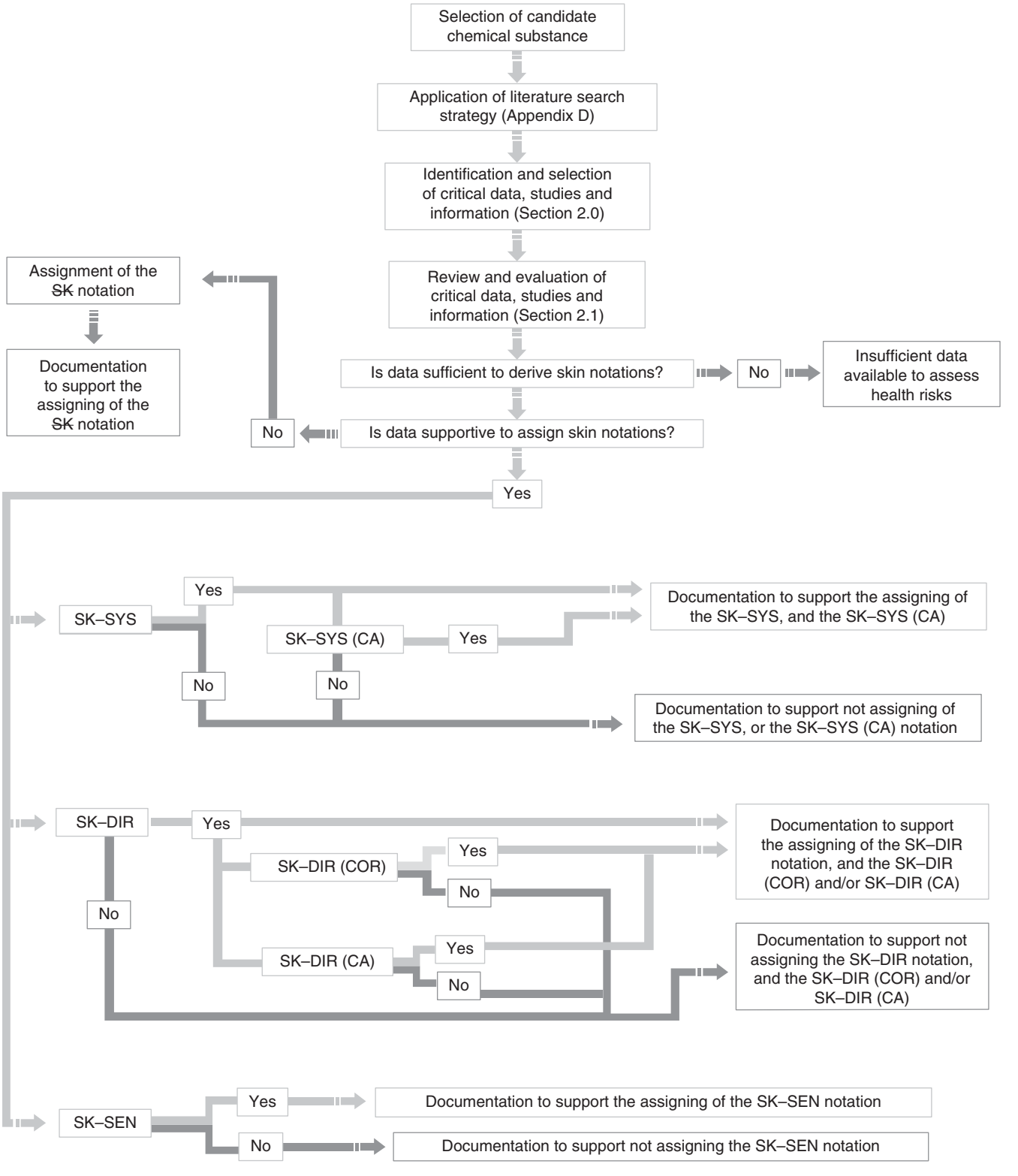

FIGURE 4.2 Schematic representation of the US NIOSH strategy of assessing skin notation.

It should be considered whether the NIOSH approach with time could be extended to cover also a marking for penetration enhancement. And it should be considered whether there could be introduced some kind of semi-quantitative way of signaling that not all chemicals are equally toxic despite being able to penetrate the skin. Actually some regulators base skin notation on the premise that it should add to the systemic exposure equivalent to 10% of the OEL. This means that the chemical with the lowest OEL will also have the lowest threshold for having *S*. But this inherent/original premise in assigning *S* seems to have questionable validity because a wide range of *S* are added based on far less rigorous rules. Therefore, the original quantitative element in *S* assignment appears partly invalidated, and a new semi-quantitative way to inform about severity may need to be developed. A semi-quantitative

information will in no way replace formal risk assessment, but may serve as a primary indicator to the manager responsible for deciding which chemicals to use, and to the worker having potential dermal exposure. This approach should incorporate variations in toxicity as well as penetration rates, which will allow a rating of *S*, while risk assessment includes an assessment of exposure, which will vary over time as well as between scenarios. *S* should relate to the potential for toxicity following relevant dermal exposure and be generic for the chemical/product in question. A refined *S* may incorporate the degree of toxicity and the dermal penetration rate.

In the Evaluations and Predictions of Dermal Absorption of Toxic Chemicals (EDETOX) EU project supported by the Fifth Framework Programme of the EC (QLRT-2000–00196), an *S* system was proposed where the degree of hazard was determined from two different types of information on the specific chemical: (*i*) percutaneous penetration and (*ii*) toxicity. A tentative algorithm was suggested for calculation of a semi-quantitative *S*, which could form a starting point for an improved *S* system (33):

$$S = 2 \times \mathrm{SYS} + \mathrm{DABS},$$

where

SYS = systemic toxicity rated from 0 to 4 based on respiratory OEL (or dermal data if available)

DABS = dermal absorption rated from 1 to 4 based on Kp values.

The range will be 1–12 and could be expressed as a traffic lights system where 1–3 is green, 4–8 yellow, and 9–12 red.

This kind of algorithm, suggested for discussion, would be able to rate the penetration characteristics as well as toxicity profile on numeric scales allowing the calculation of an integrated number. It represents an attempt of giving a practical tool easily perceived and used by industry, particularly small- and medium-sized enterprises. The categorical approach could be integrated in an extensive strategy, such as the NIOSH system.

At this time the US NIOSH proposal is very positive because the specific preventive area deserves more attention. It also illustrates that different countries go their own ways, and the revised US NIOSH system is very different from EU approaches illustrated by the German or the SCOEL assignment criteria. Thus, the existing and significant discrepancies between *S* in different countries will remain unless individual countries to a larger extent are willing to harmonize rules and regulations in this area.

If a chemical gets the *S*, the consequence will likely include economic impact; that an alternative and potentially more expensive chemical should be used, that specific technical precaution needs to be installed to minimize the risk of skin contact, or that personal protective equipment should be used. If significant differences exist between countries, this may potentially influence industrial placement of facilities at the expense of workers' safety.

There are therefore compelling reasons for trying to develop a system to protect workers against hazardous skin exposure based on uniform and transparent methods that is used widely in as many countries as possible.

ACKNOWLEDGMENTS

We thank Hans Drexler (Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine, University Erlangen Nuremberg, Germany) and Gunnar Johanson (Work Environment Toxicology, Karolinska Institutet, Stockholm, Sweden) for their kind collaboration and the John Wiley and Sons Publication for the permission to reproduce *American Journal of Industrial Medicine* content.

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5 Skin ion channels in health and disease

Iván Restrepo-Angulo, Miriam Cortés Torres, Andrea De Vizcaya-Ruiz, and Javier Camacho

PART I: INTRODUCTION

Ion channels are pore-forming membrane proteins that allow the flow of ions down their electrochemical gradient and play major roles in cell physiology. The relevance of these proteins has been strongly stressed in pathologic conditions. Alterations in either expression or activity of some ion channels have been associated to several diseases, such as cardiac arrhythmias, epilepsy, skeletal muscle disorders, cancer, diabetes, and cystic fibrosis, among others (1). Accordingly, around 30% of the drugs available in the market target ion channels.

These proteins constitute a diverse group found in the cell membrane as well as in the membrane of intracellular compartments, including mitochondria, endoplasmic reticulum, and nucleus. Although ion channels were mainly associated to the physiology of excitable cells for a long time, now it is known that they participate in the physiology of very different cell types, including epithelial cells, leukocytes, glial cells, and spermatozoa (2,3). The role of ion channels is not merely devoted to their function as pores allowing the flow of ions; they can also activate enzymes linked to cellular signaling pathways, serve as cell adhesion molecules or components of the cytoskeleton, and their activity has been shown to be related with the alteration in the expression of specific genes (4).

The effect of toxic xenobiotics on several ion channels suggests a strong association between ion channels and human toxicology. Some examples of this association are the regulation of ion channels by several chemicals found in air, water, or soil, the effect of pesticides on ion channels, the undesirable side effects of a huge amount of clinically used drugs, and targeting of ion channels by many animal toxins (5).

Membrane transporters and ion channels have been described in the skin both in normal and pathologic conditions, and might be used as either markers of exposure or therapeutic targets. Aquaporins (AQPs) and transient receptor potential (TRP) channels have been found in keratinocytes of the epidermis and melanocytes. Some potassium channels have also been reported in immortalized keratinocytes and melanoma cell lines, and potassium channel blockers have been shown to decrease the proliferation of melanoma cells.

This chapter reviews some of these proteins described in cells from the skin, their association to some skin diseases and the regulation of skin ion channels by toxicologic agents. We will start describing some of the most common membrane transporters and ion channels reported in keratinocytes and melanocytes, namely, TRP channels, AQPs, and acetylcholine receptors.

TRP CHANNELS, AQUAPORINS, AND ACETYLCHOLINE RECEPTORS: AN OVERVIEW

Transient Receptor Potential Channels

The TRP channel family comprises more than 30 cation channels, most of them being permeable to Ca^{2+}. This family is divided into seven groups: *TRPC* (TRP-canonical), *TRPV* (TRP-vanilloid), *TRPM* (TRP-melastatin), *TRPP* (TRP-polycystin), *TRPA* (TRP-ankyrin), *TRPML* (TRP-mucopilin), and *TRPN* (TRP-nonmechanoreceptor potential). Four subunits with six transmembrane domains are associated to form the TRP channels (Fig. 5.1). These channels are widely distributed in many tissues, including the nervous system tissues, heart, pancreas, placenta, testis, tongue, and digestive system organs, among others (6). TRPs are regulated by many different factors, such as intra- and extracellular ligands and mechanical, osmotic, and chemical stress as well as temperature (7). Another very interesting feature is that some of them are activated by calcium depletion from intracellular pools (8). Due to their expression in epidermal cells, it is important to mention some features of the members TRPV1–4, TRPM8, and TRPA1.

TRPV comprises four groups of mammalian channels: TRPV1/TRPV2, TRPV3, TRPV4, and TRPV5/6. The members 1–4 show a permeability ratio P_{Ca}/P_{Na} between 1 and 10; on the contrary TRPV5 and TRPV6 are highly permeable to calcium. TRPV1 is gated by a variety of stimuli, including vanilloid compounds, such as capsaicin, resiniferatoxin (these two substances in a highly specific manner), olvanil, as well as moderate heat (≥43°C) and low pH (≤5.9). TRPV2, which exhibits a 50% sequence identity with TRPV1, is activated by noxius heat (≥53°C), insulin growth factor-1 (IGF-1), and neuropeptide head activator (6). Interestingly, TRPV2 is phosphorylated and modulated by protein kinase A (PKA) in cutaneous mast cells (9). TRPV3 is activated by innocuous warm temperatures (>30–33°C) and by camphor, which is a natural compound widely used in medicine. TRPV3 can co-assemble with TRPV1 to form functional hetero-oligomeric channels. TRPV4 can be activated by diverse stimuli, including heat (>24°C), cell swelling, shear stress, anandamide, some metabolites of arachidonic acid, and the non–PKC-activating α-phorbol ester, 4α-phorbol 12,13 didecanoate (4α-PDD). TRPV4 exhibits multiple putative consensus sites for PKC (6). TRPM8 is primarily permeable to calcium. It is activated by cold temperature (8–28°C) and pharmacologic agents evoking cool sensation, such as menthol and icilin in a pH-dependent fashion. The calcium influx through TRPM8 induces activation of phospholipase C, leading to depletion of phosphoinositide 4,5 P2 and a

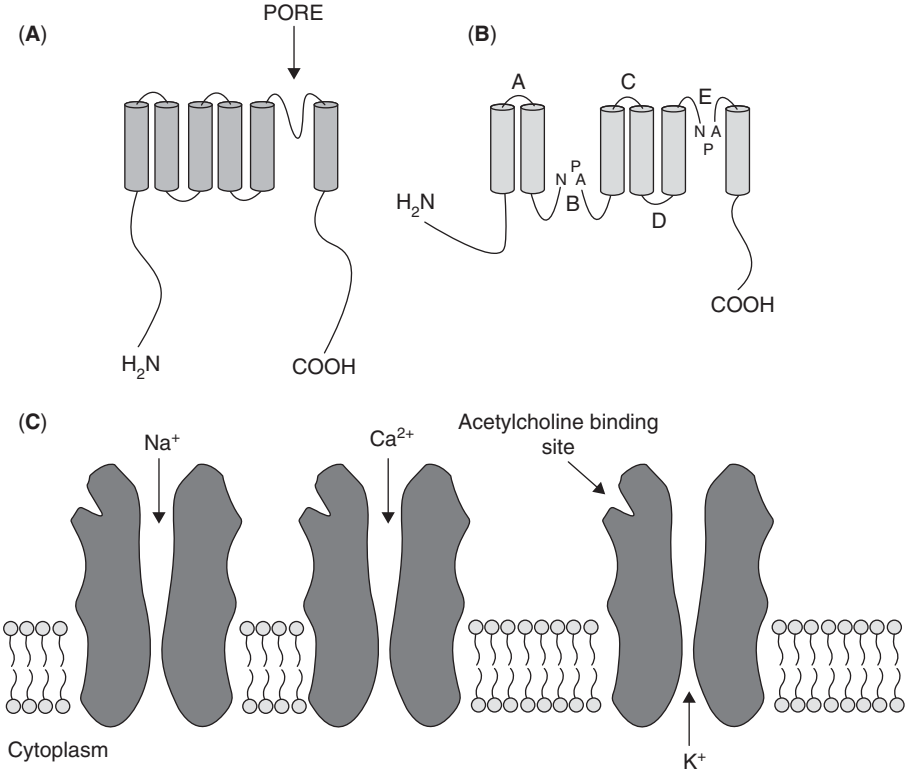

FIGURE 5.1 Schematic structure of TRP, aquaporins, and acetylcholine receptors. (**A**) TRP subunits are composed by six membrane-spanning domains. Four subunits are required to form the functional channel. As in voltage-gated ion channels, the pore is found between the fifth and the sixth transmembrane segments. (**B**) Aquaporins have six membrane-spanning α-helices with N- and C-termini located on the cytoplasmic side. Hydrophobic loops B and E have the highly conserved sequence asparagine-proline-alanine (NPA) in which the asparagine residue is essential for the pore water-selective filter (65). (**C**) Subunits α, β, γ, δ, and ε can bind each other to form the nAchR. Selectivity of nAchR depends on the combination of subunits forming the channel (13). *Abbreviations*: nAchR, nicotinic acetylcholine receptor; TRP, transient receptor potential.

consequent decline in channel activity. Finally, TRPA1 exhibits 14 N-terminal ankyrin repeats, which may be involved in channel mechanosensitivity. Its function is calcium- and voltage-dependent, although it is gated by isothiocyanates, tetrahydrocannabinoid, cinnamon, and bradykinin. It is also activated by noxious cold (6).

TRPM channels have been grouped on the basis of sequence homology as follows: TRPM1/3, TRPM2/8, TRPM4/5, and TRPM 6/7. These channels show varying permeability to divalent cations, from highly permeable to calcium and magnesium to completely impermeable to calcium (TRPM4 and TRPM5). TRPM1 (melastatin), is the founding member of the TRPM family. It has been proposed to be a constitutively open calcium channel. Several studies have focused on this channel due to its role as a tumor suppressor protein (6).

Aquaporins

Aquaporins (AQPs) are membrane proteins that form water-selective pores primarily facilitating osmotically driven water transport across the cell membrane. Data from electron and X-ray crystallography show that AQP monomers contain six membrane-spanning helical domains surrounding a narrow aqueous pore (Fig. 5.1). AQP monomers are superassembled in the membrane as tetramers (10). There are at least 13 mammalian AQPs (AQP0–AQP12), which have been divided into two groups on the basis of their permeability. AQPs 1, 2, 4, 5, and 8 are primary water-selective pores. AQPs 3, 7, 9, and 10 transport either water or glycerol. It has also been reported that AQPs may transport gases and ions across the membrane. AQPs are involved in several physiological functions, such as regulation of urinary concentration, neural signal transduction, and epithelial fluid secretion (11). Importantly, it has also been proposed that AQPs promote cell migration by enhancing water transport in lamellipodia at the leading edge of migrating cells (12).

Acetylcholine Receptors

Muscarinic (metabotropic) and nicotinic (ionotropic) acetylcholine receptors have been found in the human skin. Metabotropic acetylcholine receptors are G-protein coupled receptors that play an important role in cardiac physiology. On the other hand, human nicotinic receptors (nAChR) are composed of different subunits: α1–α10, β1–β4, γ, δ, and ε, which can bind each other to form either homo- or heteropentameric channels. For example, the α1, β1, and δ subunits form heteropentamers present at the neuromuscular junction together with γ and ε subunits; the later found in adults (13). Nicotine receptors are permeable to potassium, sodium, or calcium depending on the subunit composition (Fig. 5.1) (14). The composition of the channel is also a pivotal feature modulating the affinity for acetylcholine and different agonists, such as nicotine and choline.

PART II: SKIN ION CHANNELS AND AQUAPORINS IN HEALTH AND DISEASE

Table 5.1 summarizes some examples of the potential role and effect of ion channels and transporters in skin cells in health and disease.

TABLE 5.1
Channels and Transporters in Skin Cells

Channel	Cell Type	Agent Involved	Effect/Role	References
TRPV1	Dorsal root ganglion sensory neurons	Histamine Eicosanoids	Pruritus	(20)
TRPA1	Dorsal root ganglion sensory neurons	Chloroquine Methyl isocyanate Hexamethylene diisocyanate	Pruritus Skin irritation Skin irritation	(23)(29)
AQP3	Keratinocyte	All-trans retinoic acid Ultraviolet	Cell proliferation Skin dryness and photoaging	(50)(52)
TRPM1 (downregulation)	Melanocyte	—	Cell proliferation	(59)
Human nicotinic receptors	Fibroblast	Nicotine	Aging	(13)
K^+ channels (Eag1 and K_{Ca})	Melanoma cells	—	Cell proliferation	(64)

TRP CHANNELS IN NORMAL AND PATHOLOGIC SKIN

Thermosensation

TRPV channels have been detected both in afferent fibers from the sensory neurons innervating the epidermis of the skin and in keratinocytes. It is widely known that TRPV1–4 channels in nervous fibers participate in sensing thermal stimuli. Observations from TRPV3-null mice suggest that these channels have a role in thermosensation in keratinocytes. In mice, TRPV3 channels are not found in neurons of the dorsal root ganglion (DRG) but in keratinocytes (15). TRPV3-null mice show deficits in its response to innocuous and noxious stimuli caused by heat, while other sensory properties were not affected. In primates, TRPV3 is found in neurons of the DRG; therefore, further studies are necessary to determine the role of keratinocytes in thermosensation and the hypothetical role of TRPV3 in such process (16). TRPV3 may also be involved in the development of some skin pathologies. Mice bearing a Gly573Ser substitution, which increases the activity of the channel, develop allergic and pruritic dermatitis. These mice show an increased innervation of afferent fibers, high levels of the nerve growth factor in response to heat, and an exacerbated scratching behavior (17).

Histamine and Chloroquine-Induced Pruritus

TRPV1 is also expressed in keratinocytes and it has been proposed to be involved in pruritus. TRPV1 is activated by pruritogenic substances and mediators of itching, including eicosanoids, histamine, bradykinin, and ATP. Capsaicin, an active compound from chilli that inhibits the activity of TRPV1 channels, suppresses histamine-induced itching and it can be used in pruritus treatment (18). TRPV4 is expressed in keratinocytes and seems to be involved in the development of eczema. Interestingly, extracts from the Chinese medicinal herb *Andrographis paniculata*, which has been traditionally used for this pathology, enhances the activity of the TRPV4 channel (19).

Histamine released from immune cells, such as mast cells is a well-known inducer of itching. It has been shown that histamine induces severe itching when applied to the skin experimentally. This effect is supposed to be mediated by the histamine receptor 1 (H1), which is expressed in the sensory afferents that innervate the skin. H1 is a G-protein-coupled receptor, which enhances the accumulation of arachidonic acid via the activation of phospholipase A2 (PLA2). 12-Hydroperoxyeicosatetraenoic acid (12–HPETE) is produced by the metabolism of arachidonic acid and is able to induce the activity of TRPV1. This signaling pathway suggests a link between TRPV1 channel activity and itching (20,21). In support of this observation, when 100 µg of histamine was added to cultured rat neonatal dorsal root ganglion neurons (DRG neurons), small inward currents were activated in a small group of cells; such currents were abolished by capsazepine (10 µM), an antagonist of capsaicin, which inhibits the activity of TRPV1. In addition, histamine evokes calcium currents in DRG neurons, which are dependent on extracellular calcium. These currents were dramatically reduced in DRG neurons from TRPV1-knock out mice. The scratching behavior induced by histamine was also decreased in these mice in spite of the normal expression of the downstream effectors of the histamine signaling pathway (20).

Chloroquine (CQ) has been used in the treatment of malaria in many tropical countries for a long time. One of the major side effects of CQ is itching, which may vanish spontaneously after drug withdrawal and is not associated with skin lesions. Pruritogenic potential of CQ substantially reduces its use affecting the control of malaria (22). Pruritus induced by CQ is independent of histamine and is mediated by the members of the Mas-related-G-protein receptors (Mrgprs) (also known as Mrg/SNSR) family. Mrgprs receptors are activated by peptides with RF/Y-G or RF/Y amide ends, such as the bovine medulla peptide (BAM), the molluscan FMRFamide, and the mammalian neuropeptide FF (NPFF) among others. In DRG neurons, family members Mrgpr As, B4, B5, C11, and D are found in mice and MrgprX in humans, suggesting their involvement in somatosensation. Deletion of Mrgpr A and Mrgpr C genes in mice led to a significant decrease in the number of scratching periods induced by CQ when compared with wild-type (WT) mice. It has also been shown that CQ at micromolar concentration (297.68 ± 2.10 µM) activates the MrgprX heterologously expressed. CQ (1 mM) treatment in cultured DRG neurons induced a robust intracellular calcium increase in WT mice. Such effect was not observed in DRG neurons from Mrgpr-deficient mice. Interestingly, the intracellular calcium increase was almost completely blocked in calcium-free extracellular solution and it was impaired by ruthenium red, which is an inhibitor of TRP channels. This strongly suggests that TRP

channels are involved in the CQ-induced pruritus via Mrgpr receptors (23). In this regard, it was recently shown that TRPA1 is the downstream target of MrgprA3 and MrgprC11 in cultured sensory neurons. In addition, sensory neurons from TRPA1-deficient mice exhibited marked reduced responses to chloroquine and BAM. TRPA1-deficient mice showed either a little or no scratching behavior in response to this pruritogenic, suggesting that it may be a component in the signaling pathway that promotes histamine-independent itching (24).

Psoriasis

Psoriasis is a chronic immune skin disease characterized by inflammation, leukocyte infiltration, and enhanced keratinocyte proliferation. In in vitro and in vivo studies, the differentiation and proliferation of keratinocytes is regulated by an increase in intracellular Ca^{2+} via both Ca^{2+} release from intracellular stores and Ca^{2+} influx mechanisms. High extracellular Ca^{2+} stimulates phospholipase C pathway, which in turn can induce Ca^{2+} release from endoplasmic reticulum and consecutive Ca^{2+} influx. In this process, TRPC channels have been suggested to be involved. The expression levels (mRNA and protein) of all TRPC channels are significantly reduced both in cultured psoriatic keratinocytes and psoriasis plaques. Thus, it has been suggested that TRPC channels might be a novel target for psoriasis therapy. Specific TRPC6 activation by hyperforin not only elevates Ca^{2+} but also at least partially overcomes the intrinsic defect in maturation (25).

Atopic Dermatitis

TRP vanilloid type 1 (TRPV1) is highly expressed in epidermal keratinocytes as well as in the nerve fibers distributed in epidermis and dermis; recently, Denda et al. (26) reported that TRPV1 activation in epidermal keratinocytes might be closely associated to skin barrier disruption.

In an AD in vivo model, it was demonstrated that blockage of TRPV1 activation by TRPV1 antagonists could accelerate the recovery from skin barrier damages and suppress the development of AD-like symptoms, such as elevated IgE in serum, mast cell degranulation, scratching behavior, and skin inflammation (27). It is suggested that this effect is mediated by the increased Ca^{2+} influx in keratinocytes following TRPV1 activation and the subsequent perturbation of epidermal barrier maturation. Calcium influx into epidermal keratinocytes affects lamellar body exocytosis and consequently delays the recovery from barrier disruption (26).

Industrial Isocyanate and Volatile Organic Compounds

Isocyanates, a group of reactive chemicals compounds used extensively in the production of polyurethane foams, coatings, and a wide array of consumer products, have been associated to asthma worldwide. Spraying and application of foams and adhesives provide the opportunity for skin to be exposed to isocyanate from depositions of aerosols and/or absorption. Animal studies using radiolabeled 14C methyl-ENE-diphenyl diisocyanate (MDI), the major commercial isocyanate, have shown absorption of MDI after skin exposure. Hexamethylene diisocyanate (HDI)-conjugated keratines have been identified in skin biopsies obtained after epicutaneous application of HDI, indicating skin absorption. Allergic contact dermatitis has been reported following skin exposure and it has been also observed in animal models (28). Methyl isocyanate (MIC), a precursor of pesticides, identified after the environmental disaster in Bhopal, India) and HDI activate heterologously expressed TRPA1 channels (EC_{50} of $25 \pm 3\,\mu M$ and $2.6 \pm 0.7\,\mu M$, respectively). These compounds induced a calcium increase in DRG neurons; such effect was not observed in TRPA1-deficient neurons. Nociceptive responses, such as licking after intraplantar injection of HDI (6 mM) were reduced in mice treated with HC-030031, a blocker of TRPA1. A similar behavior was observed in TRPA1-deficient mice. These results support the role of TRPA1 in sensing irritating stimuli (29). Further studies are required to determine the role of TRPV1 in allergic contact dermatitis induced by industrial isocyanates in humans.

Diverse volatile organic compounds, including toluene, xylene, and formaldehyde, irritate the skin and cause allergic and neurogenic skin inflammation. A recent study demonstrated that TRPV1, expressed in keratinocytes, is involved in the vascular hyperpermeability induced by volatile organic compounds, demonstrating that this channel might be either an initiator or enhancer of formaldehyde-induced skin inflammation in mouse (30). Thus, TRP channels seem to play important roles in chemically induced skin toxicity.

Ultraviolet Light and Aging

Skin aging can be attributed to intrinsic and extrinsic processes. Extrinsic aging is generally referred to as photoaging because it is most commonly caused by repeated exposure to ultraviolet (UV) light. Alterations in collagen, the major structural component of the skin, have been considered to be a cause of skin aging but the mechanisms of collagen destruction in aged skin have not been fully clarified. Collagen destruction is partly related to the induction of matrix metalloproteinases (MMPs), a family of structurally related matrix degrading enzymes that play important roles in various destructive processes. MMPs are secreted by epidermal keratinocytes and dermal fibroblasts and their levels are increased by various stimuli, such as UV light, oxidative stress, and cytokines (31).

Recently, it has been suggested that calcium can regulate the expression or activation of MMPs. Increased extracellular calcium levels induce the MMP-9 gene expression in human keratinocytes (32,33) and the inhibition of calcium influx decreases the level of MMP-1 mRNA (34). Modulation of intracellular calcium levels can modify the secretion of MMP-1 from migrating keratinocytes (35). In previous studies, it was suggested that UV light activates TRPV1 with the subsequent increase in $[Ca^{2+}]$-induced MMP-1 in human keratinocytes, so epidermal TRPV1 may function as a sensor of UV light (36).

AQUAPORINS IN NORMAL AND PATHOLOGIC SKIN

Hydration and Wound Healing

Several AQPs have been found in keratinocytes. AQP9 mRNA was only detected in differentiating keratinocytes (37), and AQP10 was detected in primary keratinocytes cultures from human skin (38). AQP3 is the most abundant AQP in the skin. It was first detected in rat epidermis and then in the basal layer of keratinocytes in humans and mice (11). It has been proposed that AQP3 has a very important role in skin hydration, and in migration and proliferation of keratinocytes (39). The skin of AQP3 knock out mice was dry, rough, aged, and showing low levels of glycerol

when compared with WT mice (40,41). In addition, keratinocytes from these AQP3-null mice showed poor permeabilities to water and glycerol (11). These results suggest a pivotal role of AQP3 in the transport of glycerol and consequently in the maintenance of skin hydration. In addition, AQP3-null mice, display a delayed wound-healing process (41). Probably, AQP3 promotes water influx, which is required to form protrusions at the leading edge of migrating cells. It was observed that migration of cultured human keratinocytes is impaired with small interfering RNA (siRNA) targeting AQP3; such treatment mimics the behavior of keratinocytes from AQP3-null mice (11). In vitro scratch assays showed that the wound closure was also delayed in keratinocytes from AQP3-null mice and reduced membrane protrusions were observed in the wounding edge. Thus, suggesting participation of AQP in skin hydration and wound healing.

Hydration, Retinoic Acid, and UV Radiation

All-trans retinoic acid (ATRA) and its derivatives, commonly called retinoids, exert potent keratolytic effects on the skin and are frequently prescribed in severe forms of psoriasis (42). Retinoids also inhibit sebocyte proliferation and differentiation, and have therapeutic value for the treatment of acne vulgaris (43). Moreover, retinoids are potent stimulators of keratinocyte proliferation (44) through the release of heparin-binding epidermal growth factor produced by suprabasal keratinocytes (45), and inhibit the expression of key genes involved in keratinocyte differentiation, such as caspase 14 or differentiation-specific keratins (46,47). By regulating keratinocyte proliferation and differentiation, retinoids increase stratum granulosum thickness and are widely used in cosmetics for the treatment of skin aging (48,49).

Since glycerol transport through AQP3 is involved in skin hydration and elasticity, several cosmetic products in the market contain ingredients that enhance the expression of AQP3 (10). For example, retinoids are widely used in cosmetics for the treatment of skin aging. It has been shown that ATRA (1 μM) significantly increases AQP3 expression after 2 hours of exposure. Significant accumulation was observed after 3 hours and 24 hours of incubation and AQP3 basal levels were restored after 48 hours in a concentration-dependent manner. Application of an oil-in water carbopool-based emulsion containing 0.05% ATRA also increased the mRNA and protein levels of AQP3 in skin explants (50).

On the other hand, UV radiation, which is one of the most relevant extrinsic factor contributing to skin photoaging, downregulates AQP3 expression in cultured keratinocytes (HaCat cells). Interestingly, inhibitors of MEK/ERK, such as PD98059N and U0126, inhibit UV-induced AQP3 loss (51). The effect of UV on AQP expression is also counteracted by ATRA. In addition, pretreatment with ATRA attenuates reduced water permeability, decreased cell migration, and delayed wound healing induced by UV. It has been shown that the protective effect of ATRA is mediated by the epidermal growth factor receptor and that it involves the inhibition of MEK/ERK (52).

Tumorigenesis

Although AQP3 can be targeted in the development of improved cosmetic products as well as in new therapies of skin disease associated with altered skin water content (11), there should be a caution in the use of such enhancers of the AQP3 expression due to the association between this water channel and tumorigenesis (53). At least 13 tumor cell types have been found to express various AQPs. AQP expression correlates with tumor aggressiveness in some tumor types. Water transport through AQPs has been associated with tumor angiogenesis as well as to migration, invasiveness, and cellular metastatic potential. As mentioned above, AQP3 has been involved in skin tumorigenesis as it has been found overexpressed in skin squamous cell carcinomas. In support of this hypothesis it has been shown that AQP3-deficient keratinocytes have impaired cell proliferation compared with that in WT keratinocytes. Accordingly, AQP3-null mice did not develop skin tumors after exposure to phorbol esters, whereas WT mice developed multiple tumors (11). It has been proposed that AQP3-glycerol transport is a determinant of epidermal cell proliferation and can induce tumorigenesis by a novel mechanism in which glycerol would be a key regulator of ATP energy (11).

Arsenic Transport

Arsenic is a ubiquitous environmental toxicant, it has been classified as group A human carcinogen and its major source is the drinking water. The two major oxidation states in inorganic arsenic are trivalent arsenite (As^{III}) (the more toxic form) and pentavalent arsenate (As^{V}) (oxidized but a less toxic form) (54). Zebra fish is an excellent model to study arsenic-associated diseases, it is a vertebrate with rapid embryonic development and organ differentiation, and its entire genome has been sequenced. Many zebrafish genes exhibit high sequence similarity to their human homologs. In a recent study, Hamdi et al. proposed that aquaglyceroporins have the ability to transport metalloids, such as arsenite into several zebra fish tissues, such as eyes, gill, intestine, kidney, liver, and skin (55). Experiments of arsenic exposure on toad skin show that arsenic toxicity affects the activity of transporters and channels, including the sodium pump, sodium conductance, ionic passive conductance, as well as the ability to maintain the electric parameters needed for normal skin physiology (56). Although these results suggest that AQPs and channels are involved in arsenic toxicity, further studies are needed to corroborate its participation in human skin toxicity.

ACETYLCHOLINE RECEPTORS IN NORMAL AND PATHOLOGIC SKIN

Differentiation

The non-neuronal cholinergic system has been implicated in several functions of skin cells, such as growth, differentiation, adhesion, motility, and barrier formation, as well as in pathologic conditions, such as acne vulgaris or atopic eczema.

Several studies have shown the expression of the α3, α5, α7, α9, α10, β2, β4, and β1 nAChR subunits in human skin. Differences in the expression could be a result of a variety of factors, such as age, atopic disposition, smoking habits, or minimal trauma. Subunits α3, α5, and β2 have been found in the basal layer and in the stratum granulosum. The α9 subunit has also been detected in the basal layer in significant levels, whereas the α7 subunit is found in the stratum granulosum and spinosum co-localizing with α10 and β1. Blocking all nAChR with mecamylamine and atropine or strychnine (which blocks α9) in organotopic co-cultures for 7–14 days resulted in inhibition of epidermal differentiation and proliferation, and in intracellular lipid accumulation. It should be highlighted that blockage of nAChR with

mecamylamine led to less pronounced differentiation and proliferation than the blockage of muscarinic receptors with atropine. Decreased expression of adherent- and tight-junctions and subsequent apoptosis was also observed after treatment with AChR blockers. In contrast, stimulation of nAChR and muscarinic receptors with cholinergic drugs resulted in thickened epithelium and an increase in the intracellular lipid content. It was also shown that acetylcholine is necessary for keeping or maintaining stratified epidermis-like epithelium in vitro, suggesting that it might protect keratinocytes from apoptosis (13).

Cell Cycle-Related Proteins and Extracellular Matrix Remodeling

Human dermis fibroblasts express α3, β3, β4, α5, and α9 nAChR subunits (57). In vitro exposure of dermis fibroblasts to nicotine is associated with the expression of p21, cyclin D1, Ki-67, caspase 3, and Bcl-2 mRNA transcripts. This effect is blocked by mecamylamine, suggesting the participation of nAChR α3 subunit in the nicotinic control of dermis fibroblast. In accordance, α3-null mice show decreased mRNA levels of p21, Ki-67, cyclin D, and bcl-2, and increased levels of p53 and caspase-3. Interestingly, fibroblast from α3-null mice show an increase in the mRNA and protein level of the metalloproteinase-1 (MMP-1) and the protein level of collagen Iα1. On the contrary, mRNA and protein of elastin were found to be decreased. These findings propose a role for nAChR in the extracellular matrix remodeling properties of dermis fibroblast (13).

Skin Aging Induced by Smoking

Skin acetylcholine receptors may also provide an explanation about some effects of tobacco use in human skin (13). It has been proposed that smoking can be an etiologic factor for premature skin aging (i.e., dry, thin, pale, and rough skin, among others). Long-term exposure to nicotine affects nAChR subunit gene expression, as well as the composition of nAChRs, and ligand-binding kinetics, modifying nicotinic pharmacology of the exposed cells. Moreover, desensitization of nAChR by nicotine-induced overstimulation may lead to an antagonist-like effect. It has been suggested that the nAChR-mediated regulatory pathway may be involved in the early appearance of premature aging in skin tobacco users.

ION CHANNELS IN MELANOMA

TRP Channels

TRPM1 (a nonselective ion channel initially named melastatin) is likely the most well-studied ion channel in melanocytes. It has been hypothesized that TRPM1 plays a role in melanocyte pigmentation. In human neonatal epidermal melanocytes, TRPM1 levels correlate with melanin content (58). It has also been observed that melanocytes lacking TRPM1 channels show a decreased tyrosine activity and intracellular melanin pigmentation (59). Interestingly, TRPM1 is downregulated in human melanoma and regional or complete loss of TRPM1 mRNA in primary cutaneous melanoma correlates with an increased risk of developing metastatic disease (60,61).

In the murine melanoma cell lines B16, TRPM1 show the lowest mRNA expression in the B16-F10 cell line, which has highly metastatic potential. On the contrary, this channel has a higher expression on melanoma cell lines that show a poor metastatic ability. Melastatin expression in human formalin-fixed neoplasic tissue was found in benign melanoma samples and was only found in cells with melanocytic differentiation. In addition, it has been shown that TRPM1 expression is low in rapidly proliferating melanocytes compared with the slowly growing, differentiated melanocytes (59). The above-mentioned expression patterns have been suggested as indicators of melanoma aggressiveness (62).

Potassium Channels

Calcium-regulated and voltage-gated potassium channels have been found in different types of human melanoma cell lines. The kinetics of the major component of the potassium currents found in the melanoma cell lines IGR1 and IPC298 shows the slow, voltage- and magnesium-dependent kinetics typical of the oncogenic *ether à go-go 1* (EAG1) channels (63). Incubation of IGR1 cells for 48 hours with imipramine (10–15 mM), a nonselective blocker of EAG1, induced a decrease in DNA synthesis with no significant effect on apoptosis. Other types of potassium channel blockers, such as charybdotoxin and blockers of chloride channels did not affect the proliferation of melanoma cells, suggesting that EAG1 has a primary role in melanoma cell proliferation and that this channel could be a therapeutic target for this type of cancer (64).

CONCLUSION

Ion channels play key roles in human physiology and toxicology, and are very important targets for several major diseases. Studies on the expression of membrane transporters and ion channels in skin, as well as research on ion channel regulation from exposure to toxic agents, could provide important information for a better understanding of the mechanisms underlying dermatotoxicity effects. With no doubt, membrane transporters and ion channels will offer new exposure markers and potential therapeutic targets to treat skin diseases.

ACKNOWLEDGMENTS

Investigations related to some of the topics here included have been partially supported by the CONACyT grant # 82175-M to JC.

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6 Systemic toxicity

Germaine L. Truisi, Howard I. Maibach, and Philip G. Hewitt

INTRODUCTION

Human skin is exposed to a number of chemicals and drugs throughout our entire lives. Following percutaneous absorption, a chemical and/or its metabolites may cause toxicity locally or in another organ, distant from the point of entry. Although not generally appreciated, some chemicals are more toxic, at least in animals, when applied topically rather than orally. Furthermore, many compounds are absorbed to a greater degree from the skin than the gastrointestinal tract, and whole body exposure can produce systemic absorption up to grams of material. This chapter focuses on the limited epidemiologic material available and depends mostly on case reports. Many compounds that are dermally absorbed are capable of producing systemic side effects whose occurrence and severity depends largely on the many factors that can affect the absorption of topically applied compounds (both physiologic/pathologic condition of the skin and physicochemical properties of the compound). The majority of reports for systemic toxicity have been from industrial chemicals/agrochemicals and occupationally these probably have the greatest potential hazard after dermal exposure.

FACTORS AFFECTING PERCUTANEOUS ABSORPTION

Integrity of the Barrier

The stratum corneum layer of the epidermis is a major barrier to percutaneous absorption, being more resistant toward diffusion of compounds than the layers beneath it. Therefore, anything that alters the structure and/or function of the stratum corneum will affect epidermal absorption. The integrity of this barrier is reduced by any inflammatory process of the skin, such as dermatitis or psoriasis, which may subsequently result in increased percutaneous absorption. Similarly, removal of the stratum corneum by stripping or damage by alkalis, acids, and so forth, will increase absorption as well as daily applied detergents and general hydration.

Physicochemical Properties of the Substance

Percutaneous absorption is affected by the relative water–lipid solubility of the drug and the comparable solubility of the drug in its vehicle and in the stratum corneum. In order for a chemical to penetrate through the skin into the systemic circulation it requires both a degree of lipophilicity (to facilitate its entry into the stratum corneum) and hydrophilicity (to aid its passage through the viable epidermis and dermis). Other factors, such as molecular weight, molecular volume, and melting point, are also important determinants.

Occlusion

The penetration of some compounds can be increased by the use of an occlusive covering. This can be due to increased water retention (hydration) in the stratum corneum, increased blood flow, increased temperature, or increased surface area after prolonged occlusion (skin wrinkling). Occlusion also prevents the accidental wiping off or evaporation (for volatile compounds), hence maintaining a higher local concentration on the skin surface.

Vehicle

The greater the affinity of a vehicle for the drug it contains, the less the expected percutaneous absorption will be. The physical properties of vehicles, especially the degree of occlusion they produce (e.g., greases), affects percutaneous absorption, as discussed above. Structural or chemical damage to the barrier layer can also have effect on the absorption rate; vehicles, such as dimethyl sulfoxide cause greatly increased percutaneous absorption due to stratum corneum damage (1). In general, a solvent system has a great impact on the absorption rate of a specific compound and a higher concentration of the drug in its vehicle enhances penetration. Enhanced solubility produces greater thermodynamic activity yielding greater flux.

Extensive evidence on factors affecting penetration has been published (2–4).

Anatomic Site

Percutaneous absorption is extremely variable across different body sites. Regional differences in permeability of skin largely depend on the thickness of the intact stratum corneum (5). According to the findings of a study by Feldmann and Maibach (6), the highest total absorption of hydrocortisone is that from the scrotum, followed (in decreasing order) by absorption from the forehead, scalp, back, forearms, palms, and plantar surfaces. This large variation has also been shown for certain pesticides (5). Variations in absorption with anatomic site have also been reported for other species, other than man, for example, monkey (7) and rat (8) skin.

Age

The greatest toxicologic response to topical administration has been seen in the young. The preterm infant does not have an intact

barrier function and hence is more susceptible to systemic toxicity from topically applied drugs (9,10). A normal fullterm infant probably has a fully developed stratum corneum with complete barrier function (11). Yet, topical application of the same amount of a compound to both adults and newborns results in greater systemic availability in the newborn (12). This is because the ratio of surface area to body weight in the newborn is three times that in the adult. Therefore, given an equal area of application of a drug onto skin of the newborn and adults, the proportion absorbed per kilogram of body weight is much more in the infant. Barrett and Rutter (13) and Maibach and Boisits (14) provide extensive documentation on this issue. Although counterintuitive, absorption of some compounds decreases in the aged (15). Later Roskos and Maibach (16) reported that absorption was decreased in older subjects for steroids, but unchanged for other, more hydrophilic compounds. They suggested that this was due to the decreased concentration of surface lipids in older subjects. Additionally, the dermoepidermal junction changes with age, so that the blood circulation of the skin decreases.

Species Variation

Mammalian skin from different species is well known to exhibit great variation in percutaneous absorption. Factors, such as stratum corneum thickness, hair follicle, and sweat gland number and the condition of the skin will play a role. The distribution of blood supply and sweating ability differs between laboratory animals and man, therefore affecting absorption through the skin (17,18).

Temperature

Generally elevated skin temperature enhances penetration rate (19). Increased temperature resulting from the exposure to heat or physical exercise involves vasodilation, which effects an increased blood flow and a rise in skin hydration (20,21).

Metabolism

It has been well documented that the skin is capable of metabolizing a wide range of xenobiotics and has a full complement of Phase I and Phase II enzymes. The specific activities found in the skin are relatively low when compared with their equivalent hepatic forms. However, when the total volume of the skin is taken into account, it is apparent that the skin is an efficient drug-metabolizing organ. This may have implications for the risk assessment of topically applied compounds, as metabolism will determine what form of the compound the systemic circulation will be exposed to. Cutaneous metabolism may also aid/impede percutaneous absorption of certain compounds. Detailed information on skin metabolism can be found in the review by Hotchkiss (22) and Zhang et al. (23).

Skin hydration, application time, concentration of the compound, particle size, solvent system, skin injuries/condition, race, sex, age, and circulatory conditions have all been reported to affect the percutaneous absorption of dermally exposed compounds.

SYSTEMIC SIDE EFFECTS CAUSED BY TOPICALLY APPLIED COMPOUNDS

Topically applied drugs, cosmetics, and chemicals can cause allergic or irritant contact dermatitis. However, this type of side effect, usually limited to the skin, is outside the scope of this chapter. The reader is referred to the textbooks of Fisher (24) and Rycroft (25) for references to contact dermatitis. Systemic side effects from topically applied chemicals can sometimes result from either a toxic (irritant) reaction or a hypersensitivity reaction. The latter can be an anaphylactic type of reaction, which is the extreme manifestation of the contact urticaria syndrome (26). Many topical drugs and cosmetics have reportedly caused anaphylactic reactions. While anaphylactic reactions to topical medicaments are uncommon, their potentially serious nature warrants attention. However, reports of toxic (as distinct from allergic) reactions to applied drugs, cosmetics, or chemicals are more numerous and include many medicaments that have been safely used for many years, but which can be toxic under special circumstances.

The following is a short summary of the chemicals that have been reported to cause systemic side effects after topical application or accidental exposure.

Agrochemicals

It has been proposed that the most serious occupational skin exposure hazard is in agricultural workers involved in pesticide application. Contaminated clothing, lack of adequate protection, and unsafe spraying procedures have caused numerous toxic responses, mainly due to skin absorption (27). Systemic toxicity after topical exposure to agrochemicals has been widely reported. A prime example is the insecticide lindane, which when absorbed into the body accumulates in the central nervous system (CNS) and the brain and has been linked with cancer (28). Therefore, the use of lindane has been restricted in the United States and has been replaced by the far safer insecticide permethrin (29); however, one case of permethrin poisoning has been reported after application to damaged skin (30). Other pesticides have been found to be genotoxic after topical exposure, including aminocarb, chlordane, DDT, and dichlorvos (31). Chlorophenoxy herbicides cause a variety of systemic toxicities, including transient gastrointestinal irritation and progressive mixed sensory–motor peripheral neuropathy (32). New guidelines have been proposed for the safety assessment of systemic toxicity caused by agrochemicals, to take into account the significant exposure after skin contact (33). In order to increase accuracy of safety assessments, not only the active ingredient must be tested but the commercially available formulation containing many function-improving additives (e.g., surfactants), which might influence the safety profile of the agrochemical. Ignorance of dermal re-entry exposures, as for example, during harvesting, may result in unforeseeable human hazard because of exposures to relevant concentrations and physical state (highly concentrated dry residue or a re-dilution of it in either human sweat or morning dew) were not examined (34). Agrochemicals such as parathion, malathion, and chlordane have been reported to persist in the skin up to 2 years after exposure (35).

Antibiotics

Chloramphenicol

Chloramphenicol, a broad-spectrum antibiotic, functions as a bacteriostatic agent by inhibition of protein synthesis (36). Oral administration of chloramphenicol may lead to aplastic anemia (37). Topical applied ophthalmic chloramphenicol formulations are systemically absorbed via the conjunctivae and the mucosae of

the nasolacrimal duct, nose, and nasopharynx (38). Marrow aplasia with a fatal outcome after topical application of chloramphenicol in eye ointment was described by Abrams et al. (39). This claim was subsequently disputed, and Walker and co-workers (40) concluded that topical chloramphenicol should not be considered to be a health hazard. Overall, a personal or family history of blood dyscrasia should be taken into account prior to chloramphenicol administration by any route (41).

Clindamycin

Topical clindamycin is widely used in the treatment of acne vulgaris. A low bioavailability was found for topically applied 1% clindamycin hydrochloride, where only 4–5% was systemically absorbed (42), and the degree of absorption largely depended on the vehicle, with values ranging from 0.1% (acetone) to 14% (DMSO) (43). Notably the systemic absorption of clindamycin phosphate is less compared with its hydrochloride salt (44). During a controlled study one treatment-related case of topical clindamycin-associated diarrhea has been reported (45). Pseudomembranous colitis is a side effect of systemic administration of clindamycin. A case of pseudomembranous colitis has been reported after topical administration by Milstone et al. (46). A more extensive overview is given by Akhaven and Bershad (47).

Gentamicin

Ototoxicity and nephrotoxicity is a well-known toxic effect of systemic gentamicin administration. However, topical application to large thermal injuries of the skin has similarly caused ototoxic effects, ranging from mild-to-severe hearing loss, with an associated decrease of vestibular function (48,49). Drake (50) described a woman who developed tinnitus each time she treated her paronychia with 0.1% gentamicin sulfate cream. Use of gentamicin-containing ear drops is associated with vestibulotoxicity if the preparation reaches the middle ear, for example, through a tympanic membrane defect (51). Accumulation in the renal cortex leads to gentamicin's nephrotoxic property (52), which might also be a risk after topical exposure (53,54). Quiros et al. (55) provide a detailed overview on the molecular mechanisms of gentamicin-induced toxicity of renal tubular cells.

Neomycin

As with all aminoglycoside antibiotics, neomycin causes renal and auditory toxicity when administered systematically. In the context of local treatment with neomycin, including skin infections and burns, (56) deafness has been reported also after application as an aerosol for inhalation, instillation into cavities, (57) irrigation of large wounds, (58) and use of neomycin-containing eardrops. (59) Kellerhals (60) reported 14 cases of inner ear damage among which the use of eardrops containing neomycin and polymycin were incriminated. All cases had perforated tympanic membranes. Neomycin's primary target is the cochlea, whereas gentamicin is considered more vestibulotoxic (61).

Antihistamines

Diphenylpyraline Hydrochloride

Diphenylpyraline hydrochloride is a histone H1 receptor agonist and has been used topically in Germany for the treatment of eczematous and other itching dermatoses. Symptomatic psychosis has been observed in 12 patients, 9 of whom were children. The amount of the active drug applied ranged from 225 to 1350 mg. The first symptoms of toxicity were psychomotor restlessness in all cases, usually within 24 hours. Other symptoms included disorientation, and optic and acoustic hallucinations. All symptoms disappeared four days after discontinuation of the topical medication (62). Other studies have shown that diphenylpyraline hydrochloride acts as a dopamine transporter inhibitor similar to cocaine (63).

Doxepin

Doxepin is a tricyclic antidepressant that in the form of doxepin hydrochloride is used for the topical treatment of eczematous dermatitis. Five percent topical doxepin has been introduced as an antipruritic. The reports of substantial drowsiness are related to overdosing the amount applied or covering the skin surface, exceeding 10% of the total body surface area (64–66). Notably Drake and Millikan (67) showed that sedation was usually transient and mild to moderate in severity.

Promethazine

Bloch and Beysovec (68) reported a 16-month-old boy treated with 2% promethazine cream for generalized eczema. The child showed abnormal behavior, loss of balance, inability to focus, irritability, drowsiness, and failure to recognize his mother. A diagnosis of promethazine toxicity through percutaneous absorption was made. Within a study comprising 15 healthy adult male volunteers, Glisson et al. (69) demonstrated that the systematic absorption of the topically applied promethazine formulation resulted in adverse reactions, which were identical to those after intravenous administration of promethazine.

Antimicrobials

Boric Acid

The toxicity of this mildly bacteriostatic substance is reviewed in detail by Stewart et al. (70). The misuse of borates has been abandoned because of their limited therapeutic value and high toxicity, resulting in few new cases of borate intoxication. Overall it was found that percutaneous absorption of boric acid through intact skin was low when compared with the average daily dietary intake (71).

Castellani's Solution

Castellani's solution (or paint) is an old medicament mainly used for the local treatment of cutaneous mycosis and also for bacterial or fungal ear infections (72,73). It contains boric acid, fuchsin, resorcinol, water, phenol (90%), acetone, and spirit and was found to be ototoxic after intratympanal infusion in laboratory animals (74). Lundell and Nordman (75) reported a case in which two applications of Castellani's solution severely poisoned a 6-week-old boy who became cyanotic with 41% methemoglobin. Another case report states that hours after the application of Castellani's paint to the entire body surface (except the face) of a 6-week-old infant for severe seborrheic dermatitis, the child became drowsy and had shallow breathing (76). Castellani's solution can no longer be used for medication due to the critical status of its ingredients (72).

Hexachlorophene

Patented in 1941 (77), hexachlorophene has extensively been used ever since, (78) mainly for reducing the incidence of staphylococcal infections among the newborn. In addition it has been an ingredient of many medical preparations, cosmetics, and other consumer goods. Hexachlorophene readily penetrates damaged skin and its absorption through intact skin has also been demonstrated (79,80). In 1972, as a result of the accidental addition of 6.3% of hexachlorophene to baby talcum powder, 204 babies fell ill and 36 died from respiratory arrest (81). This report was followed by animal experiments with hexachlorophene confirming that the drug is neurotoxic (82,83). As most chlorinated hydrocarbons, hexachlorophene holds a significant neurotoxic potential (83). Observable effects of hexachlorophene exposure include local skin reactions, such as erythema, burns, or ichthyosis, and adverse effects to the CNS, which can be life-threatening, such as convulsions, behavioral changes, CNS depression; lesions in the white matter were measured in neuropathologic assessments of laboratory animal and human material (83–85). Hexachlorophene is able to pass the placental barrier and is therefore considered embryotoxic and teratogenic (85). A study from the U.S. National Institute of Health disproved the carcinogenicity of hexachlorophene in 1978 (86). Marzulli and Maibach (87) have placed in perspective lessons to be learned from its toxicity.

Mafenide Acetate

SULFAMYLON® (Mafenide acetate, a-amino-p-toluenesulfonamide monoacetate) is a synthetic antimicrobial agent used for topical administration to treat large burns. The water-soluble drug is readily absorbed percutaneously. Due to the parent compound mafenide acetate and its metabolite P-carboxybenzene sulfonamide, both of which inhibit carbonic anhydrase, treatment may result in metabolic acidosis, which is usually compensated by hyperventilation. Notably, the inhibitory effect is amplified in patients with impaired pulmonary and renal function (88). Hyperchloremic metabolic acidosis in a 74-year-old patient with extensive burns treated topically with Sulfamylon was reported, a deterioration in renal function preceded acidosis (89). Ohlgisser et al. (90) presented two case reports (2-year-old infants) in which topical mafenide acetate administration was accompanied by methemoglobinemia that was reversible in only one case. Nowadays there exist more efficient topical antiseptics, for example, Lavasept®, however, sulfamylon treatment is considered safe, when relevant clinical parameters are monitored (91,92).

Povidone-Iodine

Povidone–iodine (Betadine) is a water-soluble complex of iodine and polyvinylpyrrolidone that retains the broad-range microbiocidal activity of iodine, without the undesirable effects of iodine tincture. However, toxicity still occurs after povidone–iodine percutaneous absorption, mainly when it is used on large areas of burnt skin, in repeated applications or on neonates. Glick et al. (93) reported an iodine intoxication after continuous mediastinal irrigation with povidone–iodine. Iodine is exclusively excreted via the urine and since povidone–iodine is nephrotoxic, urinary excretion may become problematic (94). Further information can be found in the review by Postellon and Aronow (95).

Phenol

Systemic toxicities derived from phenol burns highly depend on the total body surface area affected. When areas exceeding 10% were exposed, liver and kidney dysfunction has been reported (96). In dilutions of 0.5–2%, phenol is sometimes prescribed as an antipruritic in topical medicaments, it is used for phenol face peels, and represents the main component of Castellani's solution. It is readily absorbed through the skin and has been shown to have a prolonged elimination due to extensive tissue distribution of this lipophilic compound (97,98). Exogenous ochronosis has been reported in patients who for many years treated leg ulcers with wet dressings containing phenol or used prolonged topical hydroquinone (phenol derivate) treatment of hyperpigmentation (99–104). Several case reports document fatal reactions to percutaneously absorbed phenol: by accidental spillage of phenol (105), due to treatment of burns with a phenol-containing preparation (106), and to the application of phenol to wounds (107). A one-day-old child died after application of 2% phenol to the umbilicus (108). Several cases of sudden death and intra- or postoperative complications have been reported after phenol face peels (109). Major cardiac arrhythmias were noted in 10 out of 43 patients during phenol face peels (110). However, this is rather controversial and some authors feel that when the procedure is performed correctly phenol face peels are not potentially hazardous (111).

Resorcinol

Resorcinol is used for its keratolytic properties in the treatment of acne vulgaris. It is also a constituent of the antifungal Castellani's solution. Formerly leg ulcers were treated with external applications of resorcinol. It has an antithyroid activity similar to that of methyl thiouracil. Consequently, several cases of myxoedema caused by percutaneous absorption of resorcinol, especially from ulcerated surfaces, have been described (112–114). The prolonged abuse of resorcinol-containing ointments results in high doses of the substance and can induce reversible hypothyroidism (115). The impaired barrier function of ulcerative skin plays a major role, due to higher absorption rates (116). Overall, risk assessment analysis of these effects from "real-world" conditions suggest that human exposure to resorcinol is not expected to cause adverse effects on thyroid function after topical application (115).

Methemoglobinemia in children, caused by absorption of resorcinol applied to wounds, has also been reported (117). Cunningham (118) reported many cases of infant toxicity, including cyanosis, hemoglobinemia, hemoglobinuria, and methemoglobinemia. In the literature, the author found seven cases of acute poisoning in babies as a consequence of topical resorcinol application and five fatalities were recorded. Although the use of resorcinol in young children and for leg ulcers should be avoided, topical resorcinol, when used for acne vulgaris, has been reported to be safe (119).

Silver Sulfadiazine

Silver sulfadiazine is primarily intended for the control of bacterial infections in patients with second- and third-degree burns. There have been reports of nephrotic syndrome, agranulocytosis, and leukopenia following topical therapy (120–122). The sulfadiazine-induced leukopenia is at its peak within 2–4 days of starting therapy, with the leukocyte count returning to normal within 2–3 days. This incidence allows the exclusion of sepsis as an alternative cause for the observed leukopenia, because it occurs

not before five or six days after a burn (123). Several reports unveil a controversial relationship between silver sulfadiazine treatment and leukopenia, because the white blood cell depression resolves spontaneously and recovery is not affected by continuation of therapy (123,124). In addition Kiker et al. and Thomson et al. could not prove silver sulfadiazine to be the causative agent for leukopenia (125,126). Systemic silver accumulation in two patients undergoing prolonged silver sulfadiazine treatment was reported by Maitre et al. Monitoring of high silver concentrations in blood and/or urine are recommended by the author to prevent potential hepatic, neurologic, or nephrotic toxicity (127).

Aromatic Amines

Aromatic amines are known to cause a wide range of systemic toxicities, from acute hepatotoxicity to carcinogenic effects.

4,4′-Methylenedianiline and 4,4′-methylenebis(2-chloroaniline) are two widely used aromatic amines employed in the manufacture of polyurethane foams, epoxy resins, and as curing agents in rubber manufacture. These two aromatic amines are both listed in the current edition of the report on carcinogens as anticipated human carcinogens (128,129).

In a number of animal species these two chemicals have been shown to be mutagenic in vitro and carcinogenic in vivo (130,131). The induction of liver damage in rats was seen after systemic exposure, whereas rapid acetylators (N-acetylation) seem to be more prone toward diamine-induced hepatotoxicity (132). Both chemicals have been detected in the urine of factory workers (133,134), and several authors have reported extensive absorption through human and rat skin in vitro (135,136). Overall, dermal absorption and inhalation were found to be the predominant routes of 4,4′-methylenedianiline uptake, potentially leading to hepatitis (137,138).

Arsenic

Arsenic and inorganic arsenic compounds act as carcinogens in humans inducing various diseases and types of cancer (139). This metalloid has a wide range of use as a pesticide, as a chemotherapeutic substance, in lead alloys and as a constituent in consumer products. It also resembles a contaminant of the natural environment, which implicates a constant exposure for the human being (140). Long latency periods (28–41 years) are known for chronic arsenicism before cutaneous manifestations, mainly in the form of arsenical keratoses, Bowen's disease, squamous cell carcinoma, and basal cell carcinoma, arise and make long-term clinical follow-up examinations indispensable (141). von Roemeling et al. (142) reported multifocal malignancies of the bowel and bladder in a psoriatic patient treated 20 years with topical Fowler's solution (containing potassium arsenite), indicating that percutaneous absorption can also be carcinogenic. However, the severity of the reaction is directly related to the chemical form of arsenic as well as the duration and concentration to which the individual is exposed. A holistic view of arsenic, including its occurrence, production, toxicities, and molecular mechanisms underlying various diseases and/or disorders is provided by Tchounwou et al. (143).

Camphor

Camphor is a cyclic ketone of the hydroaromatic terpene group. It is an ingredient of a large number of over-the-counter topical remedies (with a camphor content set to a limit of 11% by the US Food and Drug Administration since 1982), taken especially for symptomatic relief of "chest congestion" and "muscle aches." Camphor is readily absorbed from all sites of administration, including topical application. The compound is classified as a Class IV chemical, that is, a very toxic substance. Hundreds of cases of intoxications have been reported, usually after accidental ingestion by children (144) and usually from exposure to relatively small amounts (145). Hepatotoxicity was reported after topical application of a cold remedy to the baby's chest and neck three times a day (146). In adults the systemic levels of camphor following use of a commercially available dermal patch were found to be low, even after application of an unrealistically large number of patches (147).

Cosmetic Agents

Cosmetic ingredients and fragrance materials are derived from a class of chemicals generally characterized by low toxicity. In a study by Di Giovanni et al. (148), 3500 cosmetics consumers were interviewed and it was found that of all the adverse effects caused by cosmetic use, only 4% of reactions were systemic (including headaches and nausea).

Henna dye, consisting of the dried leaves of *Lawsonia alba*, is traditionally used for centuries in eastern communities and is nowadays available worldwide for dyeing hair, decorating skin (Mehndi), and painting nails. In natural henna the coloring ingredient lawsone is a hydroxynaphthoquinone, which requires a prolonged and/or repeated application (2–12 hours) until desired coloring is obtained—lawsone is known to rarely cause allergic reactions (149). In order to (*i*) accelerate the fixing, (*ii*) obtain darker coloring (black henna tattoo), and (*iii*) expand the durability of these temporary dyes henna mixtures are increasingly adulterated with para-phenylenediamine (PPD). More than 300 cases of severe intoxication, some fatal, due to systemic exposure of PPD are reported merely at the ENT-Hospital in Khartoum every year (150). Initial symptoms include angioneurotic edema with massive edema of the face, lips, glottis, pharynx, neck, and bronchi and occur within hours of skin exposure. The symptoms may then progress on the second day to anuria, renal tubular necrosis, and acute renal failure with death occurring on the third day (151). Whether this toxicity is due to PPD per se (probably grossly impure) or its toxicity is potentiated by its combination with henna is unknown (152).

As a potent skin sensitizer, dermal exposure to PPD can cause severe allergic reactions, including contact urticaria or dermatitis and anaphylaxis. Malvestio et al. (153) showed an association with certain occupations by analyzing patch test outcomes of over 14,400 patients and their line of work. PPD penetrates the skin and is excreted, together with its metabolites, mainly via the urine, which can be used to identify cases of acute intoxication (150).

Spencer and Bischoff (154) reported that after skin penetration musk ambrette (mainly used as a fragrance) causes the breakdown of cellular elements within the brain, spinal cord, and peripheral nerves. These types of effects were also reported for the fragrance acetyl ethyl tetramethyl tetralin.

It has been suggested in the literature that certain UV filters and parabens, common cosmetic ingredient, represent a new class of endocrine active chemicals, for example, 3-benzylidene camphor and methylparaben (155–157). Up to date disruption of the endocrine system in humans by the above-mentioned chemicals has not been shown (158).

Crude Oil

Feuston et al. (159) have reported major systemic toxic effects after the dermal application of crude oils to rats. The major effects included reduction in body weight gain, increases in absolute and relative liver and thymus weight. Red blood count, hemoglobin, hematocrit, and platelet count were all affected. These effects were related to concentrations of polycyclic aromatic compounds found in the crude oil.

Human exposure to crude oil is mostly limited to occupational areas or oil spills during which volunteers are involved in the cleanup work. Ecologic damage is always well-investigated after oil spills, whereas the effect of crude oil exposure to the human health is rarely studied. Existing literature speculates that the exposure to crude oil affects human physiology and psychology, and shows genotoxic and endocrine effects (160).

Dimethyl Sulfoxide

Dimethyl sulfoxide (DMSO) is an amphiphilic molecule, frequently used as vehicle for topical pharmaceutic therapy and in in vitro studies. Its rapid skin penetration can be illustrated by a characteristic breath odor occurring after topical application. The toxicology of topical DMSO has been investigated by Kligman (161). In this study, except for the appearance of cutaneous signs as erythema, scaling, contact urticaria, stinging, and burning sensations, DMSO was tolerated well by all but two individuals, who developed systemic symptoms. In one, a toxic reaction developed that was characterized by a diffuse erythematous and scaling rash accompanied by severe abdominal cramps; the other had a similar rash, nausea, chills, and chest pain. These signs, however, abated in spite of continued administration of the drug. One fatality due to a hypersensitivity reaction has been alleged (162). Nevertheless, topical DMSO (99% solution) is being discussed for the management of cytotoxic drug extravasations (163–165).

Dinitrochlorobenzene

Dinitrochlorobenzene (DNCB), a potent contact allergen, is applied for the treatment of alopecia areata and cutaneous warts (166,167). The mutagenicity of DNCB was confirmed using the Ames test (*Salmonella typhimurium*) by different groups in the 1980s (168,169) as well its genotoxicity to in vitro human skin fibroblasts using sister chromatid exchange at lower concentrations than those applied in clinics (170). Due to the potential risks and the fact that it is absorbed in substantial amounts through the skin, with about 50% of an applied dose recoverable in the urine, (171) its use is discouraged by some physicians (172). Possible systemic reactions to DNCB have been reported. For example, a 25-year-old man treated with 0.1% DNCB (daily for 2 months) after prior sensitization, experienced generalized urticaria, pruritus, and dyspepsia (173). The immunomodulatory effects on dendritic cells of topically applied DNCB were shown to be beneficial for the treatment of patients with the human immunodeficiency virus and systemic lupus erythematosus (174–177).

Ethanol

Twenty-eight children with ethanol toxicity from percutaneous absorption were described by Gimenez et al. (178) following a popular procedure where ethanol-soaked cloths are applied to the abdomen of babies as a "home-remedy" for the treatment of disturbances of the gastrointestinal tract, or because of crying, excitability, and irritability. Ethanol-soaked cloths had been applied under rubber panties, and the number of applications varied from one to three (40 mL/application). All 28 children showed some degree of CNS depression, 24 showed miosis, 15 hypoglycemia, 5 convulsions, 5 respiratory depression, and 2 died. Of the two who died, one was autopsied and the findings were consistent with ethanol toxicity. Topically applied ethanol in tar gel- and beer-containing shampoo has caused Antabuse effects in patients on disulfiram for alcoholism, through percutaneous absorption (179,180). More recently it was found that the intended topical use of alcohol-containing cosmetic products (e.g., deodorants, perfumes) result in a very low systemic dose (181).

Fumaric Acid Monoethyl Ester

The effect of systemically and/or topically administered fumaric acid monoethyl ester (ethyl fumarate) on psoriasis was studied by Dubiel and Happle in six patients (182). Two patients who had been treated dermally developed symptoms of renal toxicity.

After topical use this substance led to persistent erythema in all healthy volunteers, which is thought to be due to mast cell degranulation (183). Furthermore, a case of contact dermatitis and one of pustulous exanthema along with systemic signs of tachycardia and dyspnea upon dermal treatment were reported (184).

Pesticides

Lindane

Lindane, the γ-isomer of benzene hexachloride, is widely used in the treatment of scabies and pediculosis. Its pesticidal action is exerted by neuronal hyperstimulation and paralysis after direct absorption into the parasites' chitin and their ova—a mechanism of action shown not to be selective for parasites. The percutaneous absorption of the drug has been widely documented (185,186), as has toxicity from excessive topical therapeutic application of lindane (187). The issue of possible toxic reactions to a single therapeutic application of lindane, notably CNS toxicity, has not been completely settled. Lindane absorption through human skin is more rapid than through animal skin. It is relatively slowly metabolized, meaning a possible accumulation and slow removal from the blood and brain (188). Therefore, safer alternatives, permethrin and malathion, have evolved as the standard care for scabies.

A summarizing overview is reported by Nolan et al. (189).

Diethyl Toluamide

N,N-diethyl-m-toluamide (DEET) has been used as the most effective insect repellent for over 50 years (190). It is commercially available in various topical forms containing between 10% and 95% DEET. Generally DEET holds low incidence of toxicity if applied as recommended; nevertheless, prolonged use in children has been discouraged because of reports of toxic encephalopathy (191). Although most reports of CNS toxicity have been in children, adults, and fetuses may also be at risk. Long-term occupational exposure led to episodes of confusion, depression, insomnia, and muscle cramps (192). Schaefer and Peters (193) reported a 4-year-old boy with mental retardation, impaired sensorimotor coordination, and craniofacial dysmorphology, whose mother

applied a lotion containing 25% DEET daily to her arms and legs throughout her pregnancy. Other systemic toxicities reported include seizures, acute manic psychosis, cardiovascular toxicity, with a few cases of death due to extensive skin absorption (194).

DEET is still the major component of topical insect repellents in the United States. Osimitz et al. (195) conclude that there is a rather low risk of severe intoxications associated with the use of DEET-containing repellents by referring to the DEET Registry, a postmarketing surveillance system, where detailed information about adverse events following DEET use is provided.

Malathion

The detailed toxicology of malathion is dealt with by Haddad (78). Malathion is used in the treatment of lice (0.5% lotion being standard). The organophosphate's mechanism of action involves the binding to the enzyme acetylcholinesterase resulting in paralysis followed by death of the lice (196). When applied according to the label, this pediculicide can generally be considered safe. Ramu et al. (197) reported four children with toxicity following hair washing with 50% malathion in xylene for the purpose of louse control. Tós-Luty et al. (198) conducted dermal toxicity studies in rats and concluded that higher doses of malathion applied dermally exerted a damaging effect on the intracellular structure of the liver, kidney, heart, and lungs.

Paraquat

The herbicide paraquat has been shown to cause genotoxicity in the bone marrow of rats after dermal application (199). It has also been shown to be genotoxic and cytotoxic to germ cells in the male rat (200).

In man reported adverse reactions upon accidental cutaneous paraquat exposure ranged from local lesions, such as burns (201,202), phototoxic contact dermatitis with toxic hepatitis (203), pulmonary fibrosis (204), hepatotoxicity (205) and even death (206).

Combination Effects

Some of these common pesticides have been studied in combination (207). Abdel-Rahman et al. concluded that real-life doses of DEET, permethrin, and malathion, alone or in combination, produce no overt signs of neurotoxicity, but do induce significant neurobehavioral deficits and neuronal degeneration in the brain of rats.

In occupational workers who were continuously exposed to a mixture of pesticides (pirimiphos methyl, chlorpyrifos, temephos, and malathion), genotoxicity, AChE activity decrease, hepatotoxcity, and nephrotoxicity was suggested to derive from the co-exposure to these chemicals (208).

Local Anesthetics

Benzocaine

Ethyl para-aminobenzoate (benzocaine) exerts its anesthetic action by decreasing the permeability of the neuronal membrane to sodium ions, resulting in an inhibition of nerve impulses. It is commonly used during inpatient and outpatient procedures, for example, endoscope intubations or teething preparations, respectively. Methemoglobinemia has been reported following the topical application of benzocaine to both skin and mucous membranes, with most cases occurring in infants (209–212). After cutaneous absorption, benzocaine promotes the generation of methemoglobin, which is incapable of binding oxygen, however, the underlying mechanism is not clear thus far. In addition, the development of methemoglobinemia upon benzocaine exposure is reported to be dependent on the susceptibility of the treated individual (213) with very young, elderly, medicated, or diseased patients being more prone (214). However, toxicity is rather uncommon.

Lidocaine

Lidocaine hydrochloride is widely used for topical, local, or regional anesthesia. Serum concentrations higher than 5 μg/mL are associated with toxicity (215). Lidocaine induces toxicity of the CNS with clinical symptoms, such as headache, vomiting, seizures, acute state of confusion, agitation, and loss of consciousness. With increasing intoxication respiratory arrest and cardiotoxicity, including reduced cardiac contraction, hypotension, and asystole have been seen (216). Systemic toxicity from lidocaine applied to the oral cavity in two children has been described (215,217). Brosh-Nissimov et al. (218) reported CNS toxicity symptoms in a healthy adult after treatment of an unusually large skin area with lidocaine-containing eutectic mixture of local anesthetics (EMLA) cream.

Mercurials

Mercury is a toxic and hazardous metal and its mechanisms of toxicity are comprehensively dealt with by Aronow (219). With few exceptions, the use of mercury in medicine is considered to be outdated. However, mercury may still be present in many drugs, even in over-the-counter formulations, and cosmetics. Metallic mercury is readily absorbed through intact skin, as are soluble and insoluble mercury compounds. After dermal absorption mercury is widely distributed in the tissues with decreasing concentration in the following organs: kidney, liver, blood, bone marrow, spleen, and intestinal wall, whereas accumulation occurs primarily in the kidneys (220). The main routes of excretion are via urine and feces. Adverse effects of the renal, gastrointestinal, and CNS systems have been reported, including reversible proteinuria, acute tubular necrosis, nephrotic syndrome, metallic taste, gingivostomatitis, nausea, hypersalivation, tremor, and neurasthenia (221,222). Young (223) examined 70 psoriatic patients treated with an ointment containing ammoniated mercury. Symptoms and signs of mercurial poisoning could be detected in 33 patients. Nephrotic syndrome has been reported after ammoniated mercury-containing ointment application (224,225). There has been a case report of a neonate who died following the treatment of an omphalocele with merbromin (an organic mercurial antiseptic) (226). In Kenya mercury-containing soap, used as skin lightening cosmetic, was found to result in clinical symptoms thought to be caused by inorganic-mercury poisoning (227).

Monobenzone

Monobenzone (monobenzyl ether of hydroquinone) is a potent skin depigmenting agent used topically to lighten residual normally pigmented skin in patients with extensive vitiligo. The underlying molecular mechanisms how monobenzone exerts its

depigmenting action includes quinone-hapten formation to tyrosinase (an important enzyme in melanin synthesis) and autophagy of melanosomes, leading to an immune response toward melanocytes (228). In 11 patients with vitiligo, on monobenzone therapy, conjunctival melanosis and pingueculae was acquired (229).

Overall, monobenzone can be considered as safe and effective (230).

Monochloroacetic Acid

Monochloroacetic acid (MCA) is used in the topical treatment of warts and for industrial purposes, such as in the synthesis of organic chemicals. This corrosive chemical has caused many fatal occupational accidents via skin exposure. Following dermal exposure MCA is rapidly absorbed and depending on the affected body area, severe systemic intoxication, even death, can be expected. In regard to the mode of action it is known that MCA causes lactic acidosis by blocking the cell energy supply and clinical effects include early symptoms, such as skin burns, vomiting followed by CNS depression, and coma. In all cases renal and cardiovascular involvement was reported, while patients commonly die because of renal failure, cardiovascular shock, or cerebral edema have also been noted (231,232). In vivo experiments in rats enabled Dote et al. (233) to conclude that the severe toxicity was probably a consequence of rapid absorption causing hepatocellular injuries, renal dysfunction, dysglyconeogenesis, and perturbation of ammonia metabolism. MCA is thought to enter the trichloroacetic acid cycle and inhibit aconitase.

Naphthol

2-Naphthol (β-naphthol) is used in peeling pastes for the treatment of acne and between 5% and 10% of a cutaneous dose has been recovered from the urine of subjects (234,235). After cutaneous resorption 2-naphthol is rapidly conjugated to glucuronide and sulfate in the liver and the main excretion route of the unconjugated and conjugated compound is probably renal (236). Extensive applications of 2-naphthol ointments have been responsible for systemic side effects, including vomiting and death (237). Hemels concluded that 2-naphthol containing pastes should be applied only for short periods of time and to a limited area not exceeding 150 cm^2 (235).

Podophyllum

The toxicity of podophyllum was reviewed by Cassidy et al. (238). Although there have been a significant number of case reports describing serious neurologic illness or death following the application of podophyllum, these are generally related to its use in dermal lesions (239,240). Systemic symptoms after dermal exposure include thrombocytopenia, leukopenia, abnormal liver function, sensory ataxia, and neurologic effects. A case of suspected teratogenicity (simian crease on left hand and preauricular skin tag) has also been reported after topical podophyllum resin treatment (241). Acute anterior uveitis has also been reported following topical podophyllum use (242).

Retinoic Acid

Retinoic acid (vitamin A) is a known teratogen when administered systematically for the treatment of vitamin A deficiency (243,244). Tretionin (all-trans-retinoic acid) is limited to topical applications due to its low oral therapeutic index (245). Overall, precutaneous tretionin absorption was shown to range from 0.5% to 7%, suggesting implausibility of systemic toxicity from its topical application (246).

Salicylic Acid

The general toxicology and percutaneous absorption of salicylates is reviewed by Proudfoot (247). Salicylic acid (SA) is widely used in dermatology as a topical application for its keratolytic properties and salicylate poisoning after topical use has been reported. An unpublished review by the U.S. Department of Health, Education and Welfare, quoted by Rasmussen (11), revealed 13 deaths associated with the widespread use of SA preparations, 10 in children. Von Weiss and Lever (248) reported 13 deaths resulting from intoxication with SA following application to the skin and several nonfatal intoxications. The most dramatic account is that of two plantation workers in the Solomon Islands, who were painted twice daily with an alcoholic solution of 20% SA involving about 50% of the body. The victims were comatose after 6 hours and dead within 28 hours (249). A case of SA toxicity leading to coma in an adult patient with psoriasis, who had been treated with 20% SA in petrolatum, was also described by Treguer et al. (250). Metabolic acidosis and encephalopathy has also been reported after percutaneous absorption of SA (251–253). Chronic SA exposure can also cause systemic inflammatory response syndrome (254). Overall, salicylate intoxication from topical treatment with salicylic acid remains rare. Nonetheless, poisoning should be considered in patients subjected to higher absorption rates (e.g., due to extended application areas, greater salicylic acid concentration, diseased skin, age) (255).

Selenium Sulfide

Selenium sulfide represents the active ingredient in antidandruff formulations also showing antimitotic activity. Absorption through healthy skin is reported to be very low, while lesions may dramatically enhance dermal uptake (256). Ransone et al. (257) reported a case of systemic selenium toxicity (tremor, lethargy, abdominal pain, and vomiting) in a woman who had been shampooing her hair two or three times weekly for eight months with a selenium sulfide suspension. Genotoxicity evaluation has revealed selenium sulfide to be have a weak mutagenic potential (258). In the 12th Report on Carcinogens, selenium sulfide was anticipated to be a human carcinogen (259).

Silver Nitrate

Ternberg and Luce (260) observed fatal methemoglobinemia in a three-year-old girl suffering from extensive burns, and who was treated with silver nitrate solution. Another case of methemoglobinemia due to silver nitrate therapy was caused by intravenous methylene blue injection in a 12-month-old child (261). Due to the hypotonicity of the silver nitrate dressings, hyponatremia, hypokalemia, and hyperchloremia may develop, especially in children (262). Excessive use of silver-containing drugs has led to local and generalized argyria and to renal damage involving the glomeruli with proteinuria (263,264). Beside the fact that silver holds antimicrobial activity, it may be toxic to various host cells (265). In an in vitro study using human keratinocytes and fibroblasts the cytotoxic effect of silver nitrate was reported at lower concentrations than those applied to patients (266).

Steroids

Corticosteroids

Topically applied corticosteroids are absorbed through the skin (267), resulting in sufficient quantities in the systemic to replace endogenous synthesis. Systemic side effects of topical corticosteroids occur more frequently in children than in adults and in patients with liver disease due to reduced metabolism of the drug (268,269). The two main consequences of systemic side effects are hypercorticism leading to Cushing's syndrome and suppression of the hypothalamic–pituitary–adrenal axis (270). Over the last 35 years as many as 43 cases of iatrogenic Cushing's syndrome due to topical steroid therapy in children and adults were published (271). In rats topical application of several corticosteroids caused body weight gain suppression, total cholesterol and triglycerides were increased, and the lymphatic tissue was atrophic. In addition, two corticosteroids caused adrenal and renal lesions (272).

Sex Hormones

Topical application of estrogen-containing preparations leads to resorption of these hormones and therefore systemic estrogenic effects. Beas et al. (273) reported seven children with pseudoprecocious puberty due to an ointment containing estrogens. The most important clinical signs were intense pigmentation of mammillary areola, linea alba of the abdomen and the genitals, mammary enlargement, and the presence of pubic hair. Three female patients also had vaginal discharge and bleeding. After discontinuation of the drug, all symptoms progressively disappeared in every patient. Pseudoprecocious puberty has also been observed in young girls after contact with hair lotions and other substances containing estrogens (274). Gynecomastia has been reported in young boys and men (275–277). A 10-month-old and two-and-a-half–year-old infant experienced precocious puberty after secondary contact to a topical testosterone formulation, which was given to the father (278,279).

Miscellaneous

There are many other examples of systemic toxicity caused by absorption through the skin. For example, exposure to acrylamide dust in polymer factories, causing a chronic disease of the nervous system (280). The neurotoxicity of acrylamide is marked by ataxia, skeletal muscle weakness, and numbness of hands and feet (281). Skin exposure to ethylene glycol dinitrate (nitroglycol) during dynamite production results in toxic effects after only a few minutes (282). Nitroglycol-related onset of symptoms (i.e., headache, nausea, vomiting, hypotonia, tachycardia, heart attack) is also known as the "Monday disease," since symptoms typically occur after re-exposition to the chemical, which after a weekend off work happened to be on a Monday (283). Carbon tetrachloride and 2-chloroethanol cause adverse systemic effects upon cutaneous exposure (284) with carbon tetrachloride known to induce hepatotoxicity and hepatocarcinogenicity (285). Glycol ethers, in particular, ethylene glycol monoethylene ether, are teratogenic and cause menstrual disorders in women (286). Mint et al. (287) showed that repeated dermal exposure of rats in vivo to dibutyl phthalate caused significant hepatic peroxisome proliferation within 14 days.

Transdermal systems contain an excess amount of drug to maintain the needed concentration gradient for drug delivery. Upon removal, patches still retain a substantial amount of active drug, (288) increasing the risk of toxicity if applied to the skin of an infant or young child, and emphasizing the need for proper use and disposal of transdermal drug-delivery systems.

Monoethanolamine, diethanolamine, triethanolamine (TEA) are industrial chemicals and the principal route of exposure is through the skin. Systemic effects after two-year TEA dermal administration in rats included hyperplasia of the renal tubular epithelium and small microscopic adenomas (289).

COMMENT

This chapter summarizes literature citations and the basic aspects of percutaneous penetration, to alert the reader to the potential for systemic toxicity from topical exposure. From the information provided in this chapter the reader can clearly see that systemic toxicities are important considerations for a diverse group of compounds after skin exposure. The severity of these systemic reactions is often worse in young children/infants or in patients with impaired barrier function (i.e., due to increased absorption).

Demonstrating causal connection (rather than association) requires careful documentation. Combining knowledge of the inherent molecular and animal toxicology, cutaneous penetration, and metabolism with the adverse human reaction, literature permits a more precise determination of causality. The above data focuses the need for controlled studies on the toxicity of chemicals, which come into contact with the skin, either accidentally or deliberately. There are many other texts emphasizing current approaches and technology (2–4,290).

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7 Chemical respiratory allergy: Opportunities for hazard identification and characterization

Elena Gimenez-Arnau

INTRODUCTION

Respiratory allergy, typically characterized by asthma and rhinitis, is an important health problem of high incidence and prevalence (1). Occupational asthma is the most common workplace lung disease in industrialized countries. Up to 20% of adult-onset asthma is caused by occupational factors. Occupational rhinitis is up to three times more frequent, and occurs together with asthma frequently (2,3). Identification of compounds having the potential to act as respiratory allergens is, in consequence, a very important and challenging area of research for industrial toxicologists.

Respiratory allergy is a hypersensitivity reaction, or immunotoxicity response, of the upper and lower respiratory tract to a protein or to a chemical xenobiotic. The reaction is immediate, with clinical features occurring within minutes to hours after xenobiotic exposure, including wheezing, breathlessness, bronchoconstriction, tightness in the chest, and in extreme cases elicit hypotension and life-threatening anaphylaxis.

Compounds inducing respiratory allergy can be classified into high (HMW) and low molecular weight (LMW) agents. HMW allergens (>1000 Da) are able to interact with the immune system directly to provoke the immune response. These include proteins encountered in the environment and/or occupationally (e.g., pollen, animal dander, enzymes) (4). In contrast, LMW sensitizers (<1000 Da) are too small to be recognized by the immune system directly and act as haptens, needing to react with carrier proteins to become immunogenic. The present chapter focuses mainly on the LMW chemical respiratory allergens.

Compared to the several hundred chemicals that are known as skin sensitizers giving allergic contact dermatitis (ACD) (5,6), there are far fewer chemicals that are known to cause respiratory allergy. Although less in number, chemical respiratory sensitizers are, nevertheless, of big concern because they are associated with high levels of morbidity, having significant socioeconomic consequences (1). Although today several approaches and regulatory models for assessing skin contact sensitization and ACD exist, there are no available or widely accepted predictive test methods for respiratory allergens. This is in large part because there is not, within the scientific community, a clear consensus on the immunobiological mechanisms through which chemicals cause sensitization of the respiratory tract. Nevertheless, current improved knowledge on these mechanisms has launched research on methods for hazard characterization. In this chapter, after reviewing briefly what is actually acknowledged about the immunobiology of respiratory sensitization compared with skin sensitization, we give an overview of the current approaches, which have been proposed for the characterization of respiratory allergens.

RESPIRATORY CHEMICAL ALLERGENS

Cellular and Molecular Events Leading to Sensitization of the Respiratory Tract

The nature of the cellular and molecular events that result in the acquisition of skin sensitization is relatively well characterized (7). In contrast, the events leading to sensitization of the respiratory tract by chemicals are not completely understood and remain controversial. It is known that skin and respiratory allergy to chemicals share some general similarities in the complex cascade of chemical and biological events leading to sensitization. However, it is also known that there are differences, especially concerning the immune response. Not all compounds provoking a specific immune response have the potential to cause hypersensitivity of the respiratory tract. A large number of skin sensitizers, for example, are believed to have no respiratory sensitizing effect (8). Why chemical allergens actually behave differently? Why is that some chemical allergens preferentially cause skin sensitization and ACD, whereas others are associated selectively with respiratory allergy?

Common to respiratory allergy and ACD is the development of hypersensitivity in two distinct phases. First is the "sensitization" phase, in which the immune system is primed to recognize and react to an antigen. Second is the "elicitation" phase, the clinical manifestation of allergy, in which a previously primed immune system reacts on following exposure to the same chemical. In order for an LMW chemical to act as a sensitizer, it needs first to react with proteins, generally via stable covalent binding, to form an immunogenic–antigenic complex. Protein reactivity is indeed an important step in the process as the size of the classic chemical allergen (<1000 Da) is too small to be recognized directly by the immune system (9). Such chemicals behave thus as haptens. The chemical may be inherently reactive, or may acquire reactivity at the relevant tissue site via metabolic activation (prohaptens) or autoxidation (prehaptens) (10). In the case of skin sensitization, different populations of dendritic cells (DC) present in the epidermis are able to recognize the immunogenic hapten–protein complex. Langerhans cells (LC), bone marrow–derived epidermal DC, have been classically considered the sole antigen-presenting cells responsible for the initiation of skin sensitization. More recently, it has been shown, however, that many other DC populations exist, which play critical roles in generating and regulating immune responses (11). Irrespective of their respective roles, LC and DC then initiate the immunotoxicity process. The most studied case is that of LC. After

encountering the hapten–protein antigenic complex, LC internalize it, treat it, and are stimulated to migrate from the epidermis to the regional lymph nodes via draining lymphatics (12). During this migration, LC undergo a maturation process in which they become antigen presenting cells. Finally, in the lymph nodes, LC present the processed antigen to responsive T lymphocytes. The ultimate result is the selective clonal expansion of responsive T cells and the release into the circulation of allergen-specific reactive lymphocytes. This characterizes the completion of the sensitization phase. The sensitized individual is now ready to start a hostile immunotoxicity response upon subsequent exposure to the same allergen. During the elicitation phase, the same process of skin penetration and protein reactivity is necessary. The antigen complex is internalized, processed, and presented to circulating T cells. In the skin, this results in a cascade of proinflammatory cytokine signaling stimulating the clinical expression of ACD. By comparison with skin sensitization, chemical respiratory allergens also need to gain access to viable epithelium (in the relevant target tissue), must form stable associations via covalent binding with proteins to acquire immunogenicity, and must engage and activate local DC to transport the allergen to regional nodes and present it effectively to T lymphocytes.

Despite these similarities, there are important differences between the immunologic mechanisms leading to ACD and to respiratory allergy (13,14). The evidence indicates that chemical respiratory allergens differ from contact allergens with respect to the immune response they elicit preferentially (Fig. 7.1) (15,16). In general, respiratory allergy is classified as a type I hypersensitivity reaction, normally involving the production of allergen-specific IgE antibody, while ACD is a type IV hypersensitivity reaction mediated by T cells. In both, skin and respiratory sensitization, T-cell activation plays a decisive role. In particular, both appear to exhibit a preferential activation of different subpopulations of T-helper (Th) cells, namely, Th1 and Th2 cells (17). Respiratory sensitization is associated with the preferential generation of Th2 cells, characterized by the production of high amounts of interleukins (IL)-4, IL-10, and IL-13. The production of these cytokines favors humoral immune function and the stimulation–differentiation of B cells to produce IgE. These antibodies bind to receptors on the surface of mast cells and basophils. Upon subsequent exposure to the allergen, these cells release inflammatory mediators, such as histamine, leukotrienes, and cytokines, which result in the immediate hypersensitivity of respiratory allergy. In contrast, skin sensitization and ACD are associated with the induction of Th1 cells, characterized by the production of IL-2, interferon-gamma (IFN-γ), and tumor necrosis factor-β (TNF-β). The sensitization response is associated with the generation of memory T cells, activated upon following encounter with the antigen and producing the delayed hypersensitivity reaction. Interestingly, it has been reported that IFN-γ produced by Th1 cells antagonizes Th2 cell responses and the production of IgE, and that IL-4 produced by Th2 cells antagonizes the development of Th1 cells. Consequently, not only do cytokines of each Th cell type promote the growth–differentiation of their lineage with the consequent hypersensitivity response, they also antagonize the proliferation of the other cell population as means of further directing the immune response (18).

Chemical respiratory allergens are therefore characterized by the ability to elicit preferential type 2 (Th2) immune responses that favor the sensitization of the respiratory tract with the consequent elicitation of respiratory allergic reactions (7). However, there remains still some uncertainty about a universal mandatory role for IgE antibody responses in exposed individuals. Although there is evidence for some individuals for the production of specific IgE antibody to the majority of chemicals confirmed as respiratory allergens, a number of studies have found that a proportion of symptomatic individuals with diagnosed occupational asthma lack demonstrable IgE (19–21). Another area of uncertainty is the route of exposure implicated in the development of sensitization of the respiratory tract. Inhalation exposure would seem to be the most appropriate route for the induction of sensitization. Nevertheless, there are studies suggesting that under some circumstances skin exposure may be efficient for acquisition of respiratory sensitization (21,22). Also, the local lymph node assay (LLNA) and guinea pig assays used for identifying skin sensitization hazards result in positive responses when testing respiratory chemical allergens (14). Although these studies are conducted in animals, some reports also exist on respiratory sensitization in humans after skin exposure with the allergen (23).

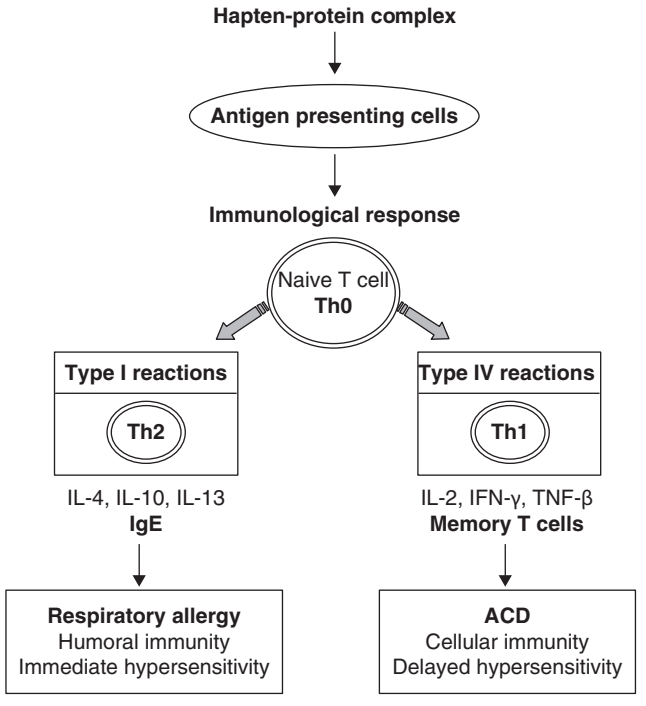

FIGURE 7.1 Principal differences between chemical respiratory and skin contact allergens with respect to the immune response elicited. The principal feature distinguishing contact and respiratory allergens is the proliferation and differentiation of T cells into Th1 or Th2 cell lineage.

Most Important Chemicals Responsible for Respiratory Allergy

As mentioned before, sensitizers that induce occupational asthma can be classified into HMW and LMW compounds. Examples of HMW agents include pollen, latex, cereals, enzymes, and a variety of animal dander, among others (24). In here we focus on LMW respiratory sensitizers. These agents behave as haptens, as they need to previously covalently bind to proteins to form the antigen responsible for the immunotoxicity response.

The most important LMW chemicals causing allergic sensitization of the respiratory tract resulting in occupational asthma are

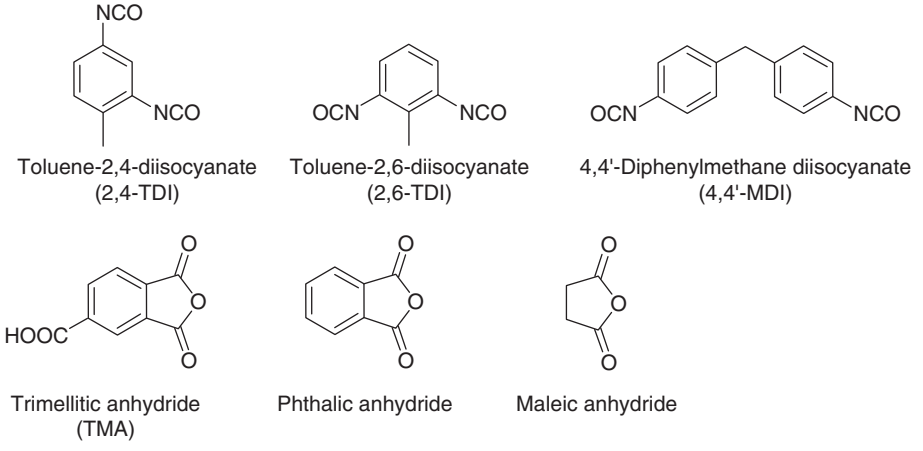

FIGURE 7.2 Chemical structures of main diisocyanates and acid anhydrides used in industry.

diisocyanates and acid anhydrides. Other chemicals associated with asthma include metals, such as certain platinum salts, pharmaceutics, and their intermediates, such as penicillin and phenylglycine acid chloride, and other industrial agents used in painting, dye making, plastics, and electronic manufacturing (25).

Allergenic diisocyanates are characterized by the very reactive –N=C=O group (Fig. 7.2). The initial step in the sensitization process is thus believed to be the covalent binding of the –N=C=O group with nucleophilic residues on proteins. Millions of tons of isocyanates are consumed annually worldwide in a wide variety of work environments. Their largest industrial use is in the manufacture of polyurethanes, employed in the production of paints, glues, plastics, surface coatings, adhesives, flexible and rigid foams, and synthetic rubbers, among many other products (26). The most commonly used isocyanates are toluene diisocyanate (TDI) and 4,4′-diphenylmethane diisocyanate (MDI). During the 1970s, TDI was predominantly used. TDI is one of the most volatile diisocyanates. Consequently, there was a shift post-1970s to the use of MDI and TDI prepolymers. Their lower volatility resulted in much lower air exposure in the workplace but, surprisingly, produced little of the expected reduction in occupational asthma (27). Also, it has been reported that exposure to MDI and TDI can cause occupational ACD, skin sensitization occurring from a single accidental exposure (28). While cases were sporadic in the 1980s, there was a clear increase in the second half of the 1990s with the increased use of polyurethanes. Both the respiratory tract and the skin are potential routes of isocyanate exposure as this can occur in the form of a liquid, vapor, or aerosol, depending on the isocyanate species and on the industrial process employed.

Acid anhydrides are characterized by the –CO–O–CO group, which can also covalently bind with nucleophilic residues on proteins (Fig. 7.2). Trimellitic anhydride (TMA) is the most important respiratory sensitizer belonging to this category of compounds. It is widely used in the industry for the production of plastics of high thermal resistance, such as polyvinyl chloride. It has been estimated that more than 100,000 tons/year TMA are produced for industrial purposes (29). On the contrary to isocyanates, TMA is known to elicit exclusively type I hypersensitivity reactions and respiratory allergy.

APPROACHES FOR HAZARD IDENTIFICATION

Given the high level of human exposure to chemicals that may cause allergic disease, there is a need to identify hazards in order to conduct accurate risk assessments to protect health. While a number of animal models and experimental approaches have been investigated for the identification of compounds with the potential to cause respiratory allergy, still today there are not accepted regulatory assays adapted to chemical respiratory sensitizers. In contrast, there are several guideline and regulatory assays for the detection of compounds with the potential to trigger skin sensitization. Given the similarities in the mechanisms of skin and respiratory sensitization, it has been proposed that assays for identifying skin sensitizers, such as the LLNA, could also be used for the detection of respiratory sensitizers, even if only a small group of the compounds yielding positive results are actually respiratory sensitizers (13). Still, due to the previously described critical differences in the immunologic responses produced by skin and respiratory sensitizers, and the more important health and regulatory implications to classify a compound as a respiratory sensitizer, it continues to be a real challenge to develop accurate assays for the identification and risk assessment of these chemicals.

Preliminary Considerations

Before to describe briefly some of the approaches that have been proposed for the identification of respiratory sensitizers, it is necessary to stress two aspects that still give some uncertainty to this research topic (30).

First is the exposure route that can result in respiratory sensitization. It has been classically assumed that the major route of exposure to chemicals leading to respiratory allergy is inhalation of the chemical allergen. However, there is now a growing agreement on the fact that also skin may be a relevant way of exposure for respiratory sensitization (21,31). Studies in animals have shown that skin exposure to TMA and diisocyanates can cause sensitization of the respiratory area in a way that further inhalation challenge with the same chemical will elicit a pulmonary reaction (32,33). This is a very important issue because it means that for successful risk assessment and prevention of respiratory sensitization in the workplace, protection from skin exposure is as important as protection against inhalation.

The second aspect is the role of IgE antibody in chemical respiratory allergy (21). Although respiratory sensitization to protein allergens and further elicitation is convincingly associated with IgE antibody, there is no clear relationship between chemical respiratory allergy and IgE antibody production. In many patients there is

confirmation for the production of IgE antibody to the majority of chemical respiratory allergens (acid anhydrides, reactive dyes, platinum salts) (34). However, it appears that there are individuals with identified occupational asthma who lack detectable IgE. Notably with respect to diisocyanates, where it has been described that less than half of those with clinically confirmed symptoms have verifiable IgE antibody (30,35). Other immunologic mechanisms could thus promote sensitization of the respiratory tract to some chemicals. Although specialists in this research area argue that the relationship occupational asthma/IgE antibody is stronger than generally acknowledged, even in the case of diisocyanates. Reasons for this are the known technical difficulties in measuring antibody production and/or the fact that serologic studies have frequently been conducted sometime after the last exposure to the inducing chemical allergen (36,37).

These unclear topics have hampered the natural wish to base methods for toxicologic evaluation and hazard identification on a solid basis of mechanistic relevance. Nevertheless, several strategies for hazard identification have been proposed for the characterization of respiratory allergens.

Brief Overview on Current In Vivo and In Vitro Approaches

Several animal models using guinea pigs or rats, but primarily the former, have been developed to investigate the pathogenesis of respiratory allergy. A detailed exposition of these models is given elsewhere (13). In here, we have given particular interest to animal models developed from the understanding of the nature of immune responses induced by chemical respiratory allergens, such as the mouse IgE test and cytokine profiling. These approaches consider what is presently understood about the mechanisms of action for contact and respiratory sensitizers with respect to IgE production and the dichotomy of the T-cell response (Th1 *vs*. Th2).

The mouse IgE test is a quantitative assay that classifies a compound as a respiratory allergen based on its ability to induce serum IgE. Technically, groups of mice are exposed topically on the shaved flanks to the test chemical (or vehicle alone). Seven days later, mice receive the same chemical at a reduced concentration on the dorsum of both ears. After further seven days, mice are sacrificed and blood drawn for measurement of serum IgE concentrations (38,39). Using this assay, many investigators have successfully reported increases in serum IgE in response to known respiratory sensitizers and not to skin sensitizers (40). However, although promising, there are inconsistencies with the approach that have limited its acceptance. For example, the known skin sensitizer dinitrochlorobenzene (DNCB) was shown to induce minimal but significant increases in IgE production, being thus a potential false positive. But also, international interlaboratory trials of the test were unsuccessful due to control housing conditions, diet of mice, and other factors that might impact the expression of IgE levels.

In parallel, many investigators have focused the attention on approaches attempting to discriminate skin/respiratory sensitizers on the basis of the cytokine profiles of Th1 and Th2 cellular responses, the so-called cytokine profiling (17). Briefly, groups of mice are exposed topically to the chemical (concentration selected upon the LLNA activity) repeatedly over 13 days. Groups of control mice are treated with TMA or DNCB. First day after final exposure, animals are sacrificed and lymph nodes excised. Single-cell suspensions of lymph node cells are then prepared and cultured for various periods of time. The production by these cells of Th1 cytokines (IL-12 and IFN-γ) and Th2 cytokines (IL-4, IL-5, IL-10, and IL-13) is finally measured by ELISA. Results with many known skin/respiratory sensitizers show in general a selective Th2 cytokine production for respiratory allergens, and a type 1 cytokine expression for skin allergens (41,42). This assay, showing that chemical allergens of different classes elicit divergent immune responses at the level of cytokine expression, is today considered by experts as a robust and reliable method of choice for the identification of chemical respiratory allergens.

Still, there is today significant social, scientific, and economic pressure to replace animal testing. The European Union ban on in vivo testing of cosmetic-toiletry ingredients came into force in 2009 (43). The REACH legislation has mandated in vivo testing to be conducted only when appropriate alternatives are not available (44). Consequently, a constant challenge is the development of in vitro approaches to reduce, refine, and replace the use of animals (3Rs). These include the use of cell culture systems and chemical reactivity assays.

Most of the research to date have focused on the development of in vitro models for the detection of skin sensitizers. These models are based on key component parts of the multistep process leading to the induction of sensitization. Thus, in vitro cell-based approaches based on cytokine measurement, changes in Langerhans cells or their equivalent, for example, have been proposed (45). In the case of respiratory sensitization, research today aims to settle in these methods for the identification of also respiratory allergens. Big progress has been made, for example, to characterize chemical respiratory allergens as a function of changes induced in DC (and dendritic-like cell lines), employing the characteristics of their maturation process, and using other cell- and tissue-based approaches (45,46). Such approaches are not yet validated and are regarded currently as being work in progress.

Additional non–cell-based in vitro approaches are based on the assessment of chemical–protein reactivity. LMW chemical sensitizers require binding to host proteins to become immunogenic. Indeed, the very first step of the sensitization process is not biological but chemical and could be used for the development of alternative in chemico methods based on the assessment of haptens reactivity (47). Chemical sensitizers often possess electrophilic properties allowing them to react with nucleophilic amino acid residues (9). This is also often the case for chemical respiratory sensitizers (48). In the context of skin sensitization, this feature has been used by many researchers exploring the use of peptide reactivity assays to identify contact sensitizers by identifying chemical–protein conjugates (49,50). In order to be able to evaluate all kinds of compounds in this kind of approach, it is important to stress that it is necessary to incorporate the fact that many skin sensitizers are inherently non-protein-reactive chemicals, and need a metabolic–enzymatic activation (prohaptens) or a chemical activation via autoxidation (prehaptens) to become reactive (10,51). The utility of this approach with respect to the identification of skin sensitizers proven, there has been interest in exploring these methodologies for the identification of chemical respiratory sensitizers and for questioning mechanistic differences that may lead to either form of chemical allergy. Our attention is focused, in next section, on the progress achieved during the last years.

Approaches In Chemico Based on Chemical Reactivity

One common approach for the identification of skin allergens based on chemical reactivity measurements focuses on incubating the test chemical in the presence of a model nucleophile and then measuring

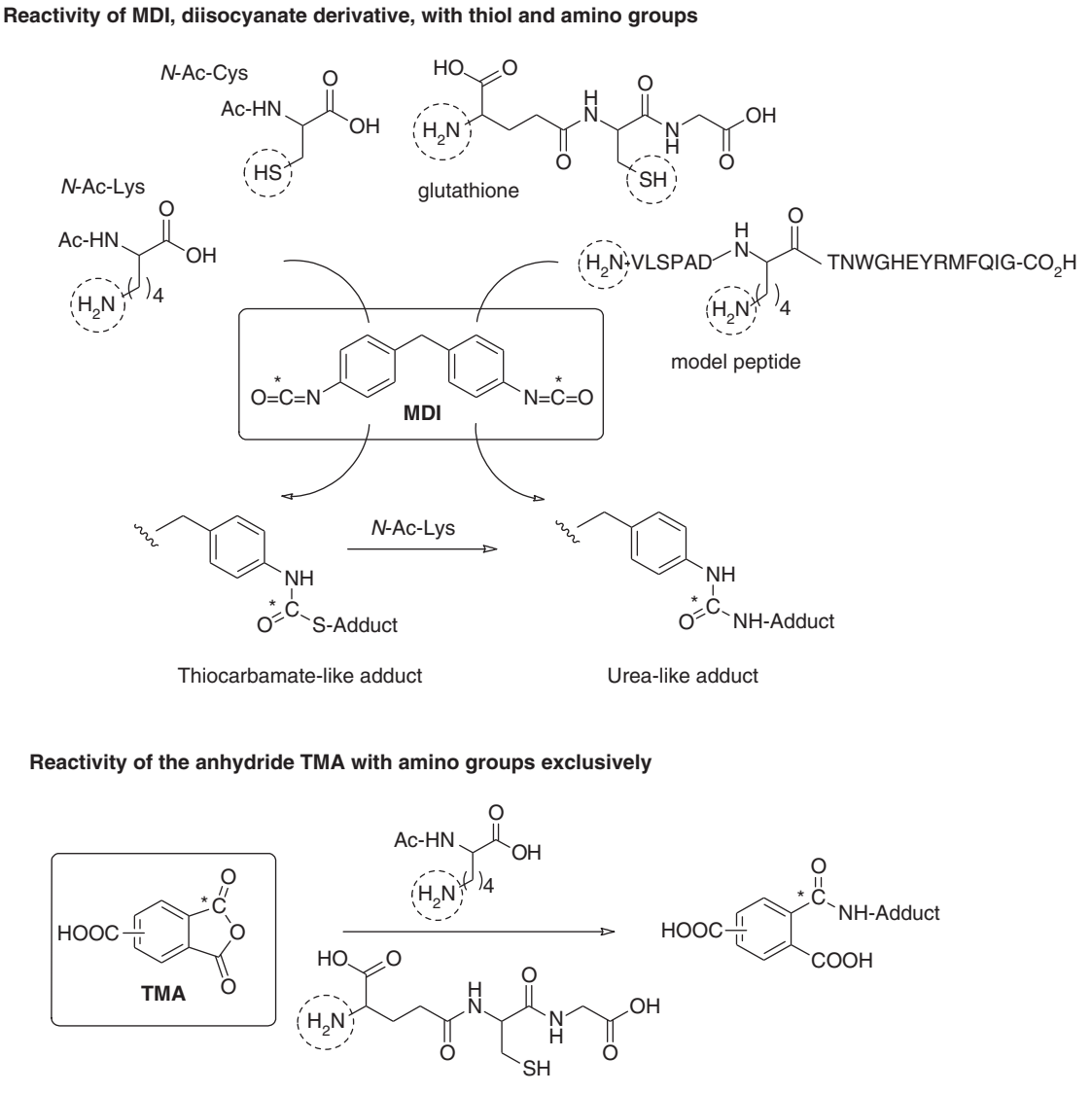

FIGURE 7.3 Reaction of isocyanates with thiol groups through the formation of thiocarbamate bonds and with amino groups to form urea derivatives (thiocarbamate adducts can further be converted into adducts of the urea kind by reaction with amino groups), and exclusive reactivity of TMA with amino groups. Studies were carried out with compounds ^{13}C labeled (*) at the reactive positions and followup of the reactivity by ^{13}C NMR.

the depletion of the nucleophile, or the formation of adducts. High-performance liquid chromatography coupled with either UV detection (HPLC-UV), or mass spectroscopy (HPLC-MS), is employed. In the case of HPLC-UV, the loss of the peptide signal is monitored and the reaction is quantitatively expressed as the percent depletion of the peptide when compared with the control (50). The loss of peptide signal is considered to be due to its modification by covalent binding with the test chemical. However, competing reactions may also take place modifying the peptide without binding of the chemical (oxidation, dimerization). To confirm adduct formation, HPLC-MS can be used, although in the most basic form it does not elucidate adduct structures (52). Nucleophiles (i.e., amino acids, designed peptides) selected until now for use in these reactivity assays are reviewed elsewhere (53). Among these approaches, one important assay is the Direct Peptide Reactivity Assay (DPRA) developed by Gerberick et al. that use heptapeptides having either a reactive cysteine or a lysine (50,54). Results with chemicals representing allergens of different potencies and nonsensitizers indicated a strong correlation between allergy potency and depletion of the peptides. The reactivity data were compared with existing LLNA potency data and a classification tree model allowing a ranking of reactivity as minimal, low, moderate, and high was built. Classifying minimal reactivity as nonsensitizers and low, moderate, and high reactivity as sensitizers, this model based on cysteine and lysine reactive amino acids gave a prediction accuracy of 89%. This assay has been further adapted for reactivity assessment of pre-/prohapten chemical sensitizers by introducing an oxidation step with horseradish peroxidase and hydrogen peroxide, the so-called Peroxidase Peptide Reactivity Assay (PPRA) (51,55).

Several investigators have included respiratory allergens in these assays. A review of representative peptide reactivity studies that consider both skin/respiratory sensitizers is given elsewhere (53). In general, the data only demonstrate that peptide reactivity is a common property of both types of allergen. However, very promising studies have shown that it may be possible to distinguish between skin and respiratory allergens by observing preferences/differences in the modification of amino acids at the protein level.

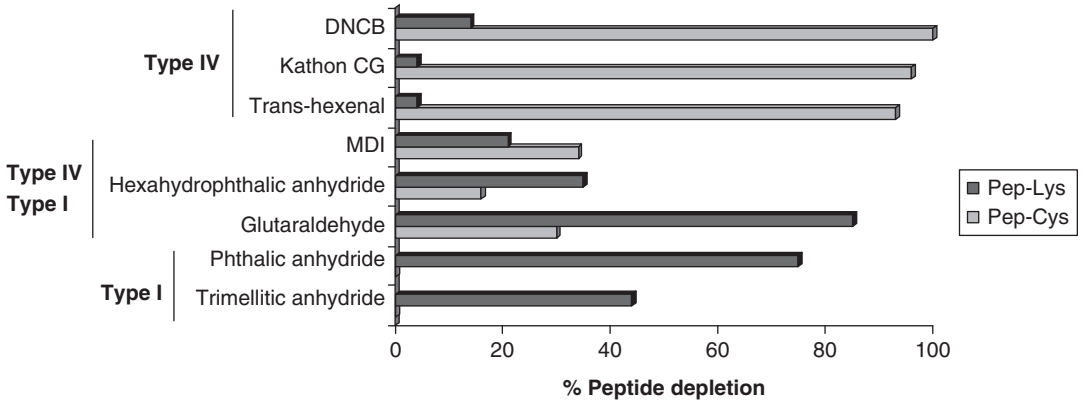

FIGURE 7.4 Direct Peptide Reactivity Assay results showing high and preferential depletion of the cysteine peptide by reaction with skin sensitizers (type IV), of the lysine peptide preferentially by reaction with pure respiratory sensitizers (type I), and of both peptides when reacting with chemicals that can produce respiratory sensitization but also skin typical immune responses (type I/type IV).

To investigate that point, in our laboratory we have looked into the reaction mechanism(s) and amino acid specificity of exclusive respiratory sensitizers, such as anhydrides, exclusive skin sensitizers taking isothiazolinones as model, and mix skin/respiratory sensitizers, such as isocyanates. On the one hand, we found that TMA, a pure respiratory sensitizer, was specifically and exclusively reacting with lysine and peptides containing lysine, while 2-methyl-2H-isothiazol-3-one, a pure skin sensitizer, was exclusively and specifically reacting with cysteine and cysteine-containing peptides. On the other hand, we found that arylisocyanates, respiratory and skin sensitizers, were reacting with cysteine and cysteine-containing peptides but also significantly with lysine and lysine-containing peptides (56) (Fig. 7.3). These qualitative results were quantitatively confirmed by the DPRA with a high lysine depletion for anhydrides, a high cysteine depletion for isothiazolinones, and a mix of cysteine/lysine peptide depletion for isocyanate derivatives (Fig. 7.4). Therefore, the DPRA could also be able to identify chemical respiratory sensitizers as it includes not only a cysteine peptide but also a lysine peptide. Moreover, it has been suggested that the depletion ratio lysine *versus* cysteine peptide could be a good approach to discriminate skin/respiratory chemical allergens.

CONCLUSION

Allergic diseases of the skin and respiratory tract resulting from exposure to LMW chemicals are of main concern for consumer product development. It is thus essential to develop available effective methods for hazard identification and risk assessment. On the contrary to skin sensitization and despite extensive research and progress on the understanding of the molecular, cellular, and immunologic events associated with respiratory sensitization and elicitation, there are still no validated or fully accepted approaches to identify compounds with the potential to cause respiratory allergy. Due to the complexity of the immune system, it has become clear that no single in vitro test will likely be adequate for hazard characterization, but that a strategic combination of simple assays covering the most important steps involved in the sensitization/elicitation mechanisms will be necessary. There is now evidence to suggest that, together with some other assays (i.e., measuring the Th1/Th2 immune response), peptide reactivity would represent a central component of any such strategy. Today, it is possible to conduct hazard identification for skin sensitizers using peptide reactivity assays. Very recent studies have suggested that it may be possible to distinguish between skin and respiratory sensitizers by using these assays, since some investigators have pointed to selective modification of specific amino acids as contributing to the ability of an allergen to act as a skin or a respiratory sensitizer. In consequence, it may be possible to distinguish between both types of allergen by observing preferences in the reactivity depending on the substrate. Progress has thus been made, and, with continued commitment and a sustained investment in research it should prove possible to develop improved tools for safety assessment of chemical respiratory allergens.

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8 Nephrotoxicity of organic solvents from skin exposure

Inge Mangelsdorf and Jens-Uwe Voss

INTRODUCTION

It has been estimated that in European companies 10 million workers are exposed to solvents (1) most of which, at least from data of animal experiments, are suspected to show nephrotoxic effects. Solvents form a structurally heterogenous group of chemicals with a widespread use for a variety of products and at different working places. Solvents are used for degreasing, dry-cleaning, and extraction of fats and oils, and can be found in a wide range of products, including paints, thinners, glues, inks, and pesticides. The largest demand for solvents comes from the paint and coatings industry, which uses almost two million tonnes every year in Western Europe, but the demand in the pharmaceutic sector is steadily growing.

Organic solvents are relatively stable volatile compounds, or mixtures of such compounds, which are liquid typically at temperatures between about 0°C and 250°C and are able to dissolve a wide range of organic compounds (2–4). This definition includes groups of liquids that may be categorized according to their chemical composition in different, somewhat overlapping groups:

- "true" hydrocarbons: aliphatic, alicyclic, and aromatic hydrocarbon compounds, mostly derived from petroleum distillation and refining;
- oxygenated compounds, e.g., alcohols, ketones, esters, ethers, glycols;
- halogenated compounds, e.g., chlorinated alkanes, such as dichloromethane, and alkenes, such as tetra- and trichloroethene;
- sulfur-containing compounds, e.g., carbon disulfide, dimethyl sulfoxide;
- nitrogen-containing compounds, e.g., dimethyl formamide.

DERMAL UPTAKE OF SOLVENTS

Most organic solvents show a high volatility at room temperature and may easily evaporate. Therefore uptake via inhalation is an important route of exposure. Additionally, dermal contact may be frequent, especially if protection is not sufficient. As many solvents may penetrate the skin (2), dermal uptake of the liquid solvents may contribute considerably to the overall exposure to solvents. In general, dermal uptake is high for compounds that are both lipophilic and hydrophilic (5). The risk of a considerable systemic exposure due to dermal uptake of a solvent (or other chemical) at the workplace is indicated by a "skin" notation in the presentation of the threshold limit value (TLV), for example, in the German TRGS 900 (6), (Table 8.1).

In the following sections, some examples are provided, where the contribution of dermal exposure to overall exposure has been determined. In general, dermal exposure to liquid has to be distinguished from dermal exposure to vapor. In the case of exposure to liquids, the mass of compound being in contact with the skin is much higher than in the case of vapor. Also, the duration of exposure is lower, coming usually from accidental short-term contact of spillages.

Liquids

For lipophilic solvents in situations with simulated accidental exposure via the skin, at maximum about the same amount may be taken up via the skin compared with uptake via the lungs (8). However, chemicals, such as glycol ethers, which provide extensive miscibility with water and common organic solvents, are taken up to a much higher degree, even if one considers that the area exposed and the exposure duration were higher in this study (Table 8.2) (8).

Vapors

For vapors, dermal exposure is less obvious. Usually, due to the high internal surface of the lung, compared with the skin, it is assumed that vapors contribute only negligible amounts to overall expose. This is, however, not generally the case (Table 8.3). For some vapors (*N*-methyl-2-pyrrolidone, *N*,*N*-dimethylformamide, the glycol ethers 2-butoxyethanol, methoxyethanol, and ethoxyethanol), dermal exposure to vapors may contribute considerably to overall exposure as has been shown in recent volunteer studies (9–12). As for dermal uptake of liquids, dermal uptake is high for solvents that are both lipid soluble and water soluble. Dermal uptake is increased with raising temperature and humidity (11). Overalls provide no protection in the case of vapor exposure (11). Physical activity reduces the percentage contribution of dermal uptake to overall uptake (10).

RELEVANCE OF KIDNEY AS TARGET ORGAN

Animal studies provide evidence that the kidney is an important target organ in general, and also after solvent exposure. Figure 8.1 shows the most relevant target organs in repeated dose toxicity studies with rats and mice. The kidney is, second only to the

TABLE 8.1
Solvents with Threshold Limit Value and "Skin" Notation According to the German TRGS 900

Substance Name	Substance Name	Substance Name
Acetonitrile	1,4-Dioxane	Methyl chloroacetate
2-Aminoethanol	1,3-Dioxolan	Methyl formate
Bis(2-methoxyethyl) ether	Ethan-1,2-diol (Ethylene glycol)	4-Methylpentan-2-on
Butanone	2-Ethoxyethanol	N-Methylpyrrolidone (vapor)
2-Butoxyethanol	2-Ethoxyethyl acetate	Morpholine
2-(2-Butoxyethoxy)ethanol	1-Ethoxypropan-2-ol	Nitrobenzene
2-(2-Butoxyethoxy)ethanol acetate	Ethylbenzene	Nitroethane
Carbon tetrachloride	Ethyl chloroacetate	1-Nitropropane
Carbon disulfide	Ethyl-3-ethoxypropionate	Oxydipropanol (dipropylene glycol)
Chloromethane	Ethyl formate	Pentan-2,4-dione (Acetylacetone)
Cumene	Heptan-2-one	2-Phenoxyethanol
Cyclohexanone	Hexan-2-one	2-(Propyloxy)ethanol
Di-n-butylamine	4-Hydroxy-4-methylpentan-2-one (diacetone alcohol)	2-(Propyloxy)ethanol acetate
1,2-dichlorobenzene	2-Isopropoxyethanol	1,1,2,2-Tetrachloroethane
Dichloromethylbenzene (ring substituted)	Methanol	Tetrahydrofurane
2,4-dichlorotoluene	2-Methoxyethanol	Tetrahydrothiophene
Diethylamine	2-(2-Methoxyethoxy)ethanol	Toluene
2-Diethylaminoethanol	2-Methoxyethyl acetate	Trichloromethane (chloroform)
N,N-Diethylacetamide	2-Methoxypropanol	Triethylamine
N,N-Dimethylaniline	2-Methoxypropyl acetate	Xylene (all isomers)
N,N-Dimethylformamide	N-Methylaniline	

Source: Data from Ref. 7.

TABLE 8.2
Comparison of Dermal and Respiratory Uptake for Some Liquid Solvents (8)

Solvent	Exposure Conditions		Dermal Uptake (% of respiratory)
	Dermal Duration Area exposed	Inhalation Duration Exposure concentration	
1,1,1-Trichloro ethane	3 min 360 cm^2 8×/8 hr	8 hr OEL	5[a]
Trichloroethene			119[a]
Tetrachloroethene			46[a]
Toluene			53[a]
Xylol			5[a]
Methoxyethanol	1 hr exposure 2000 cm^2	8 hr OEL	11[b] 100
Ethoxyethanol			2200[b]

[a]From Ref. 8.
[b]From Ref. 9.
Abbreviation: OEL: occupational exposure limit.

liver, the target organ that is most frequently affected. In 35% of all studies kidney effects are found, and in 20% effects occur at the lowest dose level tested, indicating that the kidneys are also rather sensitive to toxic injury. No major difference in target organs for systemic effects has been found between oral application and inhalation (14), and it can be assumed that also after dermal application, the kidneys are an important target organ.

The particular susceptibility of the kidney against toxic injury is related to kidney-specific physiologic features (15–20):

- A high renal blood flow: The kidneys make up only 0.5% of the human body weight yet receive about 25% of the heart minute volume. Within the kidney, the blood flows through the glomerular and the peritubular capillary system, which are more intensely exposed to blood-borne chemicals than any other capillaries in the human body;
- effective transport systems, which may lead to an accumulation of chemicals in the renal tissues, especially in the tubular epithelium;
- intrarenal fluid retention by the counterflow principle (primary urine volume: about 150–180 L/day; final urine volume: 1–2 L/day): For substances with glomerular filtration and/or tubular secretion, which are not or poorly reabsorbed in the tubules, their concentration in the tubular system compared with serum will increase several times;
- biotransformation of chemicals by xenobiotic-metabolizing enzymes, especially of the tubular epithelial cells, which may lead to the formation of toxic metabolites;
- a high oxygen and substrate demand of the renal cortex makes this region of the kidney vulnerable to substances impairing the cellular energy metabolism.

TABLE 8.3
Contribution of Dermal Uptake to Total Uptake for Different Vapors

Solvent	Exposure Conditions Activity Temperature Humidity	Part Exposed Clothing	Dermal Uptake (% of Total Uptake)	References
1,1,1-Trichloroethane	Resting 22–25°C 60–65%	Lower arm, extrapolated to whole body uncovered	0.1[a]	(13)
Trichloroethene			0.3[a]	
Tetrachloroethene			0.3[a]	
Hexachloroethene			0.1[a]	
Toluene			0.8[a]	
Xylene			0.7[a]	
Methoxyethanol	Resting 22–25°C 60–65%	Lower arm, extrapolated to whole body uncovered	55	(9)
Ethoxyethanol			42	
2-Butoxyethanol	Resting 25°C, 40%	Whole body, shorts, t-shirt	11	(11)
	Higher temperature (30°C)		14	
	+ Higher humidity (50%)		39	
	25°C, 40% Humidity	Whole body overalls	10	
N-methyl-2-pyrrolidone	Resting 23°C 39%	Whole body long trousers, cotton shirts	42	(10)
	Moderate workload		33	
N,*N*-dimethylformamide	Resting, 27°C, 44% humidity	Whole body, cotton pants, 90% uncovered	40.4	(12)

[a]%Respiratory uptake instead of total uptake.

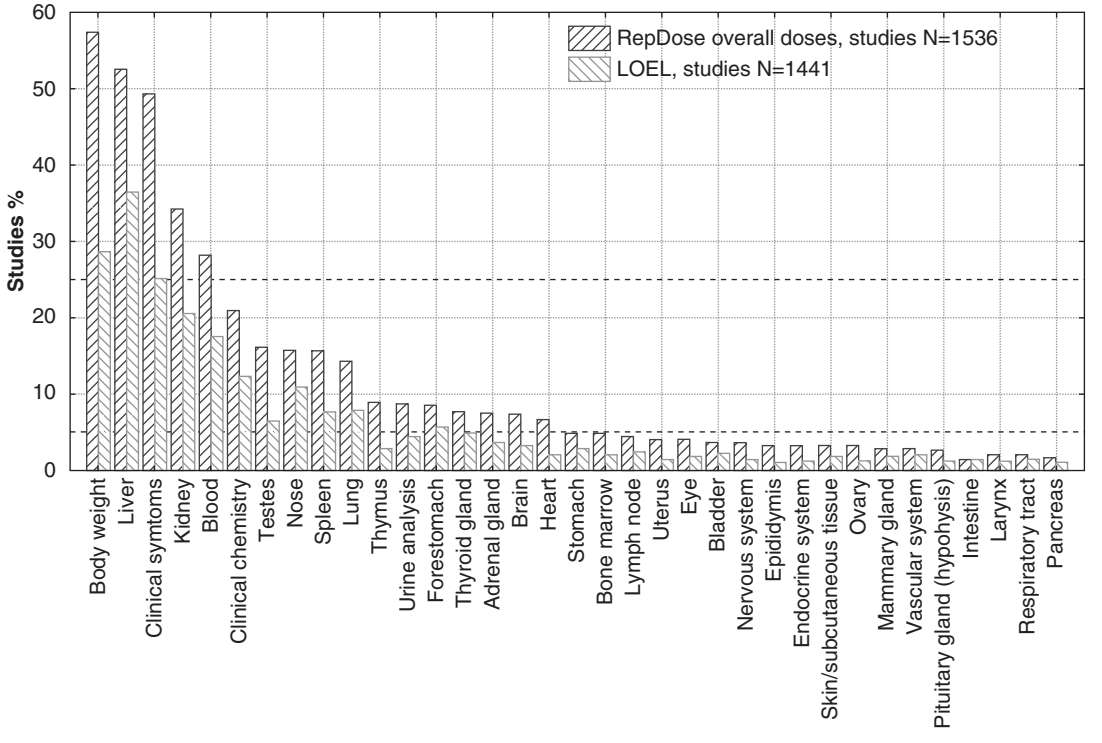

FIGURE 8.1 Targets in repeated dose toxicity studies. Species: rats and mice, Route: oral and inhalation, duration: 14 days up to 2 years, chemicals in RepDose (14). *Abbreviation*: LOEL: Lowest observed effect level.

METABOLIC ACTIVATION OF SOLVENTS AND MODE OF ACTION

Experimental animal studies have shown that a number of xenobiotics require enzymatic transformation to reactive metabolites to elicit their toxic effects in the kidney. This bioactivation may take place in the kidney or in extrarenal tissues, or extrarenally formed metabolites may be further metabolized to toxic products in the kidney. Different metabolic pathways are involved in these reactions.

The following brief overview is restricted to the most important pathways, which may contribute to the metabolism-mediated renal toxicity of solvents and illustrated by selected examples.

Cytochrome P450-mediated Bioactivation

Cytochrome P450-dependent monooxygenases are the most prominent enzymes involved in solvent metabolism. Whereas the role of hepatic cytochrome P450-mediated bioactivation of solvents in

liver damage is well studied, much less direct evidence has been presented for the involvement of the corresponding renal enzymes in kidney damage.

The activity of cytochrome P450 monooxygenases in the kidney is usually lower than in the liver. Moreover, the localization and the activity of the cytochrome P450 enzymes along the nephron are heterogenous. This may have important consequences with respect to the site of toxic action in case of solvents that require bioactivation. Spectrophotometric and immunohistochemical determinations could detect cytochrome P450 only in the cortex and the outer stripe of the outer medulla, especially in the S_2 and S_3 segments (for a schematic view of nephron architecture, see Figure 8.2) (21). The proximal tubules seem to contain the highest concentration (22). However, enzyme activity has been demonstrated also in the inner stripe of the outer medulla and the inner medulla (21).

Several isoenzymes of cytochrome P450 have been isolated and characterized from the renal cortex. The renal expression of an enzyme closely related to hepatic cytochrome P450 2E1 seems to be most relevant for the bioactivation of solvents. Immunohistochemically, gender- and species-specific differences in the activity of this enzyme have been detected—the activity in kidney of male mice being higher than in that of female mice and both genders of rats (22).

The nephrotoxicity of trichloromethane (chloroform) and of 1,1-dichloroethene (1,1-DCE) in laboratory animals represent two examples for this type of metabolic activation (21–24). Both compounds induce lesions to the proximal tubules in male mice, but less so in rats and not in female mice. Thus, the pattern of lesions coincides with the observed distribution and activity of the P450-dependent monooxygenases.

Another pathway that may lead to renal injury may involve hepatic cytochrome P450-mediated biotransformation of substances to metabolites, which are toxic to the kidney. This has been suggested as an alternative or additional pathway in the renal toxicity of 1,1-DCE.

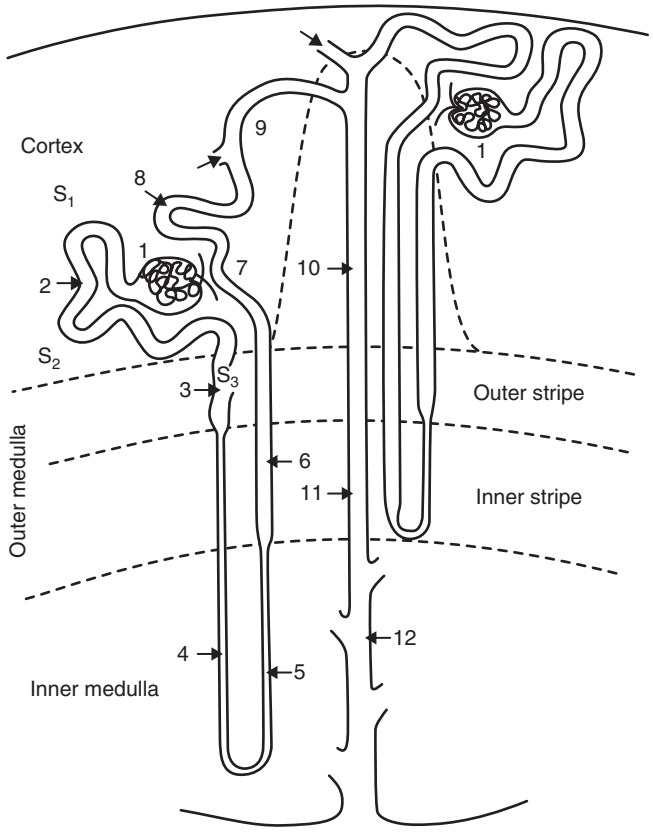

FIGURE 8.2 Schematic illustration of nephron architecture. A short- and a long-looped nephron with the collecting system are shown. 1: renal corpuscle including Bowman's capsule and glomerulus; 2: proximal convoluted tubule; 3: proximal straight tubule; 4: descending thin limb; 5: ascending thin limb; 6: distal straight tubule; 7: macula densa; 8: distal convoluted tubule; 9: connecting tubule; 10/11: cortical/outer medullary collecting duct; 12: inner medullary duct: S_1, S_2, S_3: functionally different parts of proximal tubule. *Source*: Adapted from Ref. 21.

β-Lyase–Mediated Bioactivation of Halogenated Hydrocarbons

Several halogenated alkenes are nephrotoxic in rodents. The metabolism of such halogenated alkenes is an example for a complex interaction of both inactivation and activation reactions in which both liver and kidney are involved. The current knowledge of the so-called β-lyase–mediated bioactivation has been reviewed by Dekant and Henschler (25) and Anders and Dekant (26). Briefly, nephrotoxic chlorinated haloalkenes [(e.g., hexachloro-1,3-utadiene (HCBD)] undergo glutathione-S-transferase-catalyzed reactions with glutathione (GSH). In a first step they are metabolized largely in the liver to give chloroalkenylglutathione-S-conjugates. These conjugates are excreted in the bile and pass into the small intestine. In the bile and the intestinal cells they are hydrolyzed to the corresponding cysteinylglycine- and cysteine-S-conjugates. S-conjugates may either be excreted with the feces, undergo enterohepatic circulation, or, after they passed the liver, enter the systemic circulation and are transported to the kidneys. Mercapturic acids that may have been formed in the liver also reach the kidney via the blood stream. In the kidney S-conjugates may reach the target cells in the proximal tubules via glomerular filtration and tubular reabsorption from the urine or by basolateral transport from the blood, or both. In the proximal tubular cells, due to the high activity of γ-glutamyltransferase, dipeptidase, and aminoacylase, GSH-S-conjugates, cysteinylglycine-S-conjugates, and mercapturic acids all can be broken down with the formation of the corresponding cysteine-S-conjugates. The cysteine-S-conjugates inside the proximal cells may be secreted into the blood thus undergoing renal–hepatic circulation, be acetylated and excreted as mercapturic acids (which so are both degraded and synthesized in these cells) or they may be broken down by β-lyase–catalyzed reactions. The latter results in the formation of highly reactive thioketenes, which are presumed to be the ultimate metabolites damaging the proximal tubular cells and being responsible for the nephrotoxic (and carcinogenic) effects.

It has been shown that the enzymes of the β-lyase bioactivation pathway are also present in human tissues, including those of the kidney (26). However, in vitro studies revealed that the β-lyase activity from human kidney to a number of haloalkenyl S-conjugates is several-fold lower than that from rat tissues (27,28).

The important industrial solvents tetra- and trichloroethene are also metabolized via the β-lyase pathway (21,22,26). However, in contrast to HCBD, which seems to be metabolized exclusively via GSH conjugation, tri- and tetrachloroethene are mainly metabolized by cytochrome P450; GSH conjugation represents only a minor pathway (22,29).

In conclusion, the data for the widely used solvents tetrachloro- and trichloroethene suggest that the β-lyase–mediated bioactivation

of haloalkenes is active in humans. However, quantitative species-specific differences and the contribution of other pathways seem to be important for the assessment of nephrotoxic risk of these solvents for humans (27,30–35).

Other Bioactivation Pathways

Other activation pathways, especially via the prostaglandin synthase, are important for the metabolic activation of other kidney toxins, but a role in solvent nephrotoxicity has not yet been demonstrated.

Oxidation via alcohol dehydrogenase is an important pathway for the metabolism of alcohols; with respect to kidney damage it is relevant in the special case of acute toxicity of ethylene glycol and diethylene glycol (36)

For a more detailed insight into the role of renal biotransformation, several reviews are available (21–26,29,37).

Immunologic Reactions

Based on the finding of glomerulonephritis in studies with humans, also an immune-mediated mode of action may be possible. By combining with renal proteins, hydrocarbons may act as haptens and induce autoimmunity against kidney cells. However, glomerular deposits of immunoglobulin and complement usually appeared at late stages of the disease, and deposits of immunoglobulins and complement have been seen in the glomeruli without evidence of renal disease (38).

ANIMAL STUDIES

Based on the generation of reactive metabolites in the tubuli (section – Metabolic activation of solvents and mode of action), it can be assumed that the tubuli are the primary site of toxic injury. In fact, kidneys from animals exposed to hydrocarbons frequently show tubular necrosis. Another effect occurring frequently is α2u nephropathy, a species and gender-specific effect in male rats (39).

In contrast to studies with experimental animals, in humans after chronic exposure the glomeruli seem to be most frequently affected in humans, with different types of glomerulonephritis (section – Chronic human exposure). To investigate the relevance of glomerulonephritis in experimental animals, Ravnskov (38) systematically reviewed studies with exposure to solvents. He detected 26 experiments with 13 compounds causing glomerular damage. The compounds included xylene, dibromochloromethane, trichloroethene, diacetylbenzidine, dinitrochlorobenzene, white spirit, carbon tetrachloride, trimethylpentane, petrol, maleic vinyl ether anhydride, and 4′-fluoromethylbenzanthraene. A search in our RepDose-Database (14) revealed additional five chemicals that induce glomerulonephritis in experimental animals: piperonyl butoxide, dichloroacetic acid, 5-chloro-o-toluidine, 2-propylene glycol 1-methylether, and chorothalonil. Thus, glomerulonephritis occurs in experimental animals as well and chemicals causing glomerulonephritis include important solvents, such as white spirits. All experiments resulted in various degrees of tubular damage as well.

One study was designed specifically to assess the role of styrene in the progression of adriamycin nephrosis in female Sprague–Dawley rats, a well-established model of glomerulopathy. It showed that co-exposure to styrene and adriamycin increased the urinary excretion of both albumin and low–molecular weight proteins, and was also associated with higher interstitial fibrosis score and greater cellular infiltrates as compared with adriamycin treatment alone (40). This experiment provides some evidence of the role of solvents in kidney disease progression through several potential pathogenic mechanisms, including enhanced proteinuria and tubulointerstitial damage.

ACUTE HUMAN EXPOSURE

Renal damage after acute exposure to solvents has been summarized in several reviews (24,41–51).

Solvents causing acute renal failure included 1,2-dichloropropane (52,53), trichloroethene (31,52,54,7), (53), xylene (55), petroleum naphtha (56), diesel (47,57,58), refined petrol (59), ethyleneglycol (41), and diethylene glycol (36).

Histopathologically, renal changes are confined to the tubules, and the glomeruli are intact. The tubular epithelium undergoes changes ranging from swelling, ballooning, and hydropic changes to necrosis. The tubular lumen may contain desquamated cells, pigmented casts, or amorphous eosinophilic granular material. Both the proximal and the distal tubules and the collecting tubules may be affected (42).

It is generally assumed that the acute renal failure is the result of the direct injury of the toxic compound or its metabolites on the renal tubules (49). The occurrence of tubular lesions leads to a reduction of renal cortical perfusion. This reaction may be exacerbated by a passive backflow of glomerular filtrate across the damaged tubular lumen which, in combination with the lumen obstruction by cell debris, may account for the observed oligo- or anuria (47). Another mode of action involves calcium oxalate deposition in ethylene glycol poisoning (42).

Although most case reports are from intended or accidental oral uptake, there are also some case reports with high exposure via inhalation in occupational situations. These may involve also considerable dermal contact and uptake via the skin. In one case, inhalation of C_5–C_{13} aliphatic hydrocarbons was followed by acute renal failure at two subsequent exposures (56). Three other cases occurred after respiratory or dermal exposure to diesel oil (47,57,58). In view of the widespread use of petroleum products, the scarcity of reports of acute renal failure is notable. Similarly, despite its previously widespread use as degreasing solvent in industry and in dry-cleaning, trichloroethene has only sporadically been associated with acute renal failure (31,54,7).

Although the renal changes are generally nonspecific, some solvents have been associated with certain characteristic features, for example fatty vacuolization in tetrachloromethane. The S_3 segment (Fig. 8.2) appears to be the site of nephrotoxic injury to some haloalkanes. Whether this is also true for the nonhaloalkanes remains to be determined. This segment of the nephron is rich in the inducible type of mixed-function oxidases found on the endoplasmic reticulum, which during the process of metabolism results in the formation of reactive metabolites, many of which are free radicals (24,49).

CHRONIC HUMAN EXPOSURE

Glomerulonephritis

Most studies dealing with chronic exposure have detected/investigated glomerulonephritis. The term “glomerulonephritis” (GN) is

applied to a group of diseases that are characterized by inflammatory reactions of the glomerular capillaries with cell proliferation, more or less pronounced deposition of immune complexes at the basal membrane, and often gradual destruction of the glomeruli with progressive loss of kidney function. Clinically, the most typical laboratory characteristics of most GN are glomerular hematuria with increased occurrence of dimorphic glomerular erythrocytes and reduced glomerular filtration rate (GFR) (18,60–63). Initially, a selective glomerular proteinuria (esp. albuminuria) may be found, but due to alterations of the glomerular filter's specificity and secondary tubular damage, a nonselective proteinuria may develop (18).

The most common form of GN in developed countries today is IgA nephropathy (62,64,65). IgA nephropathy (Berger's disease) results from the deposition of immunoglobulin A (IgA) in the glomeruli, where it creates inflammation. The disease was not recognized as a cause of glomerular damage until the late 1960s, when immunofluorescence techniques were applied to renal biopsies that could identify IgA deposits in kidney tissue. Although initially regarded as a benign nephropathy, end-stage renal disease (ESRD) will occur in 10−15% of all IgA nephropathy patients within a period of 15 years (66), and worldwide, IgA nephropathy is now recognized as a leading cause of ESRD in patients presenting for renal replacement therapy (65).

A rare form of a rapid-progressive GN is seen in case of a Goodpasture's syndrome. In its classical form, this is a disease affecting kidney and lung, although sometimes milder forms with minimal renal or no pulmonal involvement occur. In blood, specific antibodies against basal membrane antigen structures of the lung alveoles and the renal glomerulus are detectable. Histologically, a linear deposition of immune complexes along the basal membrane is present in the glomerulus (44,62,67–69). Other types of glomerulonephritis are membranous nephropathy and membranoproliferative glomerulonephritis (62).

Chronic renal failure (CRF) may develop from these diseases over the course of many years, as the internal structures of the kidney are slowly destroyed. In the early stage there are generally no clinical symptoms, and progression may be so gradual that symptoms do not occur until the functional capacity of the kidney is reduced to about one-tenth of normal. When a declining GFR is used as a measure of renal function and serum creatinine concentration as an indicator of GFR, a transitional stage lasting for years can be observed in many patients. During this stage, serum creatinine concentration slowly rises. Since CRF develops progressively and not stepwise, there is no clear-cut threshold for functional parameters to define the onset of CRF. Creatinine clearance is a marker frequently used with creatinine levels above 133–200 μM indicating CRF (71–75).

ESRD occurs when CRF has progressed to terminal renal failure at which the kidneys are permanently functioning at less than 10% of their capacity. At this point, dialysis or kidney transplantation is of vital importance because the kidney function is so low that otherwise complications are multiple and severe, and death will occur from accumulation of fluids and waste products in the body (63,76). Strictly speaking, ESRD is defined not only by the presence of irreversible renal failure that requires dialysis or transplantation, but also by the institution of such therapy, because patients dying of chronic uremia are not recorded as having ESRD in the existing kidney registries (77). According to Radican et al. (78) the US Renal Data System, for example, reports that in 2001 there were 406,081 cases of ESRD prevalent in the United States and over 96,000 incident cases. The disease has become more common with the age-adjusted annual incidence rate increasing nearly 53% between 1991 and 2001—from 219 to 334 per million per year. Due to the expensive treatment, ESRD represents a large and growing economic burden to the society.

Case Reports

Most case reports that describe associations of solvent exposure and glomerular damage refer to cases suffering from Goodpasture's syndrome or rapidly progressive glomerulonephritis with anti-GBM antibodies (67,68,70,79–91). A few cases of membranous GN (42,92,93) and one case of IgA-nephropathy (94) have also been associated with solvent exposure.

In contrast to the cases with acute renal failure, case reports with persons suffering from glomerulonephritis or Goodpasture's syndrome involve prolonged exposure to solvents, i.e. weeks to months (95). In most cases exposure was by inhalation, but dermal exposure may have contributed considerably, due to the type of work, for example, painting, cleaning, paint stripping.

Exposure is often not described in much detail in the case reports. Named solvents include most of the substances or mixtures that are widely used, for example, tetrahydrofurane (94), toluene (96), Stoddard solvent (83), mineral turpentine (white spirit) (84), haloalkenes and ketones (42,93), and haloalkanes (86,89), including tetrachloromethane (97). In other reports, only broad groups of agents, such as paints, thinners, degreasing solvents, or glues are mentioned (79–81,90,98). Generally, no data with respect to the presence of other possible nephrotoxic agents (e.g., heavy metals) are presented.

As case reports are not suitable to distinguish between mere coincidence or causal relationship, case reports can only be used as a starting point for more convincing epidemiologic studies.

Case–Control Studies

The case reports have prompted numerous case–control studies, that have been summarized or reviewed (3,45,47,49,51,83,99–105); most comprehensively by HOTZ (43).

The cases in these studies primarily comprised non–acute glomerulonephritides with all stages of renal function from early renal failure to ESRD. In a few studies, the case group included patients with CRF (72) or ESRD of different origin (106), various stages of diabetic nephropathy (105), or with GN related to systemic diseases (107). In most studies diagnoses were verified and specified by renal biopsy. Other occupational (72) or lifestyle factors (66) were additionally evaluated with respect to an association with the occurrence of GN.

A positive relationship between solvent exposure and nonsystemic glomerulonephritis has been found in most studies. The observed odds ratios (ORs) were higher when the rate of dropout due to death was low, the disease of the cases included in the study was more advanced, and intensity, frequency, and duration of exposure (assessed by scores) were higher. However, no specific solvents could be identified as risk factors. A meta-analysis (103) gives a significantly albeit weakly increased OR of approx. 1.6 for all studies combined.

Two studies that appeared after the review from Ravnskov (103) studies confirm the previous evaluations (108,109). However,

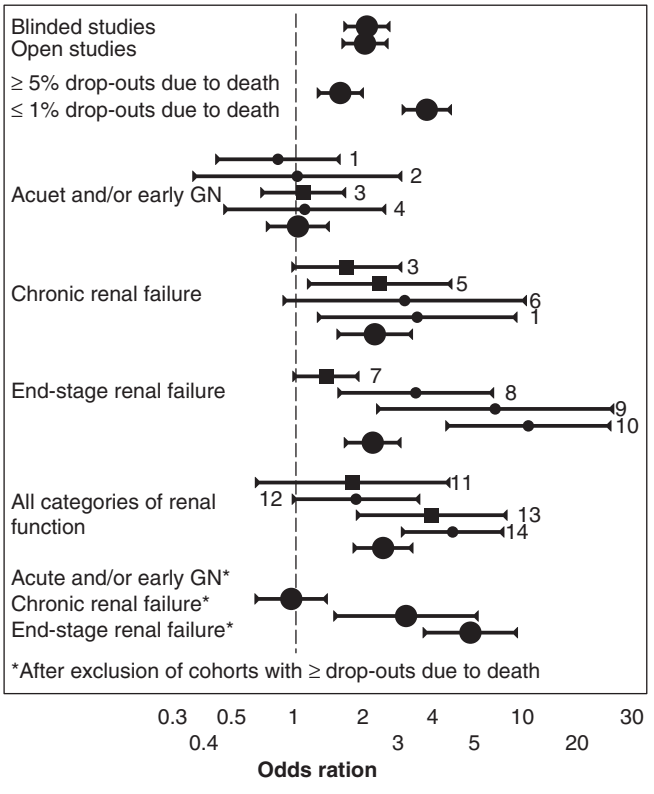

FIGURE 8.3 Graphical representation of results from meta-analysis of case–control studies. Odds ratio (OR) for exposure in individual studies: small •: OR for studies with <1% dropouts; squares •: OR for studies with ≥5% dropouts due to death before questioning; large •: mean weighted OR for various groups of studies. Bars indicate 95% confidence intervals. *Source*: Adapted from Ref. 100.

another case–control study did not find an association (110). In this study early-stage CRF from glomerulonephritis, diabetic nephropathy, renal vascular disease, or other CRF in patients with a median filtration rate of 21 mL/min were investigated. The study population was large and the authors undertook considerable efforts in assessing the exposure. No association of occurrence of renal failure with solvent exposure for any of the types of disease was found in this study and also no dose–response relationship (Fig. 8.3).

Cohort Mortality Studies

In contrast to these case–control studies, most cohort mortality studies failed to show a relationship between solvent exposure and kidney disease (43,47,111). Also a meta-analysis of 55 mortality studies on workers exposed to organic solvents was negative (112). It is likely that the study power was not sufficient for analyzing rare diseases, such as glomerulonephritis (100). Furthermore, the diagnostic categories applied in many of these studies were much too broad ("nephritis and nephrosis" or "genitourinary diseases"), including many other diseases in addition to kidney diseases, which might thus obscure a possible relationship.

In follow-up studies of the case–control studies it was observed that continued hydrocarbon exposure may be a risk factor in the progression of renal failure in glomerulonephritic patients (113,114) and in patients with diabetic nephropathy (105). This hypothesis has been tested in two recent retrospective cohort studies on ESRD.

In a huge cohort of 14,455 aircraft workers (101 total ESRD cases, 34 from diabetes, 23 from hypertension, 11 from glomerulonephritis, and 33 with unknown causes), a statistically significant increased risk of ESRD has been detected for trichloroethene, 1,1,1-trichloroethane, and JP4 gasoline (78). Their results were consistent with the hypothesis that solvent exposure aggravates or accelerates already existing renal diseases.

Another recent retrospective cohort study investigated the influence of different exposures including solvent exposure on the progression of glomerulonephritis (IgA, membranous, focal and segmental glomerulonephritis, biopsy proven) to ESRD (115,116). ESRD was defined as GFR <15 mL/(min × 1.73 m²) or dialysis. The authors found that aromatic hydrocarbons (toluene and xylene), mixtures of aromatic and aliphatic hydrocarbons, ketones, and possibly methylene chloride were associated with the highest risk. Dermal exposure was not investigated specifically in this study but rather general exposure by asking for "contact" with a given chemical. As for other studies, dermal exposure may have contributed considerably to the total exposure.

Cross-Sectional Studies

Cross-sectional studies in solvent-exposed workers have led to a complicated result (for reviews see (3,43,51,117,118)). Renal alterations, indicating effects at the tubular or the glomerular site, were seen in some studies, whereas such alterations were not found in others. A comparison of the results from the great number of studies is impossible because the study groups differed with respect to working site, type of solvents used, concentration, frequency and duration of exposure as well as parameters used to monitor renal alterations. However, in contrast to the exposed groups, signs of renal alterations were seen in control groups only in few cases.

Biomarkers of effect (for review see (119,120)), that have been analyzed most frequently in these studies included albumin, total protein, β_2-microglobulin, retinol-binding protein, and *N*-acetyl-β-D-glucosaminidase. A statistical evaluation of data from the cross-sectional studies (Table 8.4) (121) indicates that a raised albumin excretion is more frequently found in groups of workers exposed to various solvents (toluene, styrene, aliphatic/aromatic hydrocarbon mixtures, tetrachloroethene, mixtures of chlorinated hydrocarbons) than in controls. Also Green et al. (122) (a study not considered in Voss et al. (121)) reported elevated albumin and *N*-acetyl-β-D-glucosaminidase levels in workers exposed to trichloroethene.

Therefore, the study results concerning albumin are reported in more detail in Table 8.5.

In several studies no correlation with the magnitude or duration of exposure was found (122). One reason may be that short-term dermal exposure may be substantial at many solvent-exposed workplaces and may contribute considerably to overall exposure depending on the type of work and the solvent used. Although some studies mention the importance of this route (124,125), dermal exposure was not taken into account or quantified in any cross-sectional study.

Albumin is considered as marker of glomerular damage, thus an increased excretion seems consistent with the results of the case–control studies indicating glomerulonephritis as possibly associated with solvent exposure (Table 8.5).

TABLE 8.4
Explorative Statistical Analysis of the Frequency of "High" Values for Variables of Urinary Excretion in Cross-Sectional Studies with Solvent Exposure

Variable	Cutoff Limit	Frequency of Groups Containing Individuals with "High" Values		*P* value (Fisher's Exact Test, One-Tailed)
		Exposed	Non-exposed	
Albumin	37 mg/g creatinine, or 19 mg/L urine, resp.	14/14 (100%)	7/12 (58%)	0.01
	100 mg/g creatinine, or 100 mg/l urine, resp.	9/14 (64%)	2/12 (17%)	0.02
β_2M	200 µg/g creatinine, or 300 µg/L urine, resp.	7/9 (78%)	5/8 (63%)	0.4
NAG	5 U/g creatinine	7/9 (78%)	4/7 (57%)	0.4
	20 U/g creatinine	3/9 (33%)	0/7 (0 %)	0.2

High: individual values greater than some cutoff limit, where the cutoff limit has been chosen under consideration of the normal ranges given by Boege (120).

TABLE 8.5
Solvent-Exposed Groups with Individual Values of Albumin Concentration in Urine Exceeding Normal Range[a]

Industry/Work	Main Compounds	References
Oil refinery/laboratory technicians, truck drivers, bulk pant, and refinery operators	Aliphatic and aromatic hydrocarbons	(126)
Shoe production/gluers	Petroleum naphtha, toluene	(127)
Paint manufacturing and spraying	Toluene	(128)
Photogravure printing	Toluene	(129)
Floor layers	Toluene and methanol	(130)
Paint manufacturing	Xylene/toluene	(131)
Reinforced plastic industry	Styrene	(132)
Plastic boat manufacturing	Styrene	(131)
Dry cleaning	Tetrachloroethene	(133)
Dry cleaning plants	Tetrachloroethene	(134)
Dry cleaning	Tetrachloroethene	(135)
Degreasing of metals	Trichloroethene	(122)
Organochlorine plant/shift workers	Allyl chloride, epichlorhydrin, 1,3-dichloropropene, hexachlorocyclopentadiene	(136) (137)
Soil fumigation in flower bulb culture	1,3-Dichloropropene	(138)

[a]According to Boege (120): 37 mg/g creatinine or 19 mg/L.

BIOMARKERS OF KIDNEY DAMAGE

Standard metrics used to diagnose and monitor kidney injury, such as blood urea nitrogen and serum creatinine, are insensitive and nonspecific, resulting in delayed diagnosis and intervention. As demonstrated above for the cross-sectional studies, specific biomarkers may be useful for early diagnosis of kidney affections. These include several proteins, of which albumin seems to be most important.

Besides the biomarkers investigated, new biomarkers may be useful in detecting renal defects. Kidney injury molecule-1, a sensitive marker of tubular damage has been identified recently and first results indicate that it is very useful in human and in animal studies (120,139–141).

Furthermore, peptidomics in urine may be used to diagnose chronic kidney disease (142). Normal urine contains only minor amounts of peptides (22 mg peptides from 750 Da to 10 kDa/24 hours, (143)). In contrast, in the urine of patients with kidney disease peptides are found, which include fragments of different collagens, blood proteins, kidney-specific proteins, as well as fragments of various secreted proteins. While fragments from serum proteins increase, those from most of the collagens are decreased in chronic kidney disease (142). It is recommended to evaluate disease conditions not on the basis of single peptide markers, but rather on the basis of a biomarker set consisting of distinct and clearly defined discriminating molecules.

CONCLUSIONS

Despite a large number of epidemiologic studies, the issue, whether solvents cause or promote chronic kidney diseases, is still controversial. However, in our view, the weight of evidence rather supports an association. In neither type of study a significant correlation with either the magnitude or duration of exposure to the solvents was found. As solvent concentrations only are measured in air, accidental skin exposure may have contributed considerably to the overall exposure and may have obscured a dose–response relationship.

Fortunately, sensitive markers of kidney damage, such as albumin in urine (microalbuminuria), are available to detect early or moderate loss of renal function. When detected at an early stage, nephropathy may still be reversible on treatment and abatement of the exposure sources. Such markers are also useful to ensure that occupational exposure levels to potentially toxic chemicals do not entail a risk for renal function to the exposed populations (144,145).

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9 Mechanisms in cutaneous drug hypersensitivity reactions

Margarida Gonçalo and Derk P. Bruynzeel

INTRODUCTION

Cutaneous adverse drug reactions (CADRs) are considered a common problem in dermatology. They affect 2–6% of inpatients (1,2) and are a frequent cause of urgent dermatologic consultations (3). Most reactions resolve on drug suspension and symptomatic treatment but 2% can be severe with extracutaneous involvement and fatal outcome (4).

Most CADRs are certainly not immune mediated, they are expected and represent an exaggerated pharmacologic effect. They are dose dependent and occur because of increased drug bioavailability either as a result of drug interactions or concomitant diseases. Papulopustular follicular eruptions, perionychia, and hair problems occur frequently on erlotinib or cetuximab that interfere with epidermal growth factor receptor (5), and skin and oral mucosa erosions can occur with metothrexate particularly in patients with low serum albumin, low renal clearance, or on concomitant nonsteroidal anti-inflammatory drugs (NSAIDs). These predictable reactions, classified as type A, may represent up to 70–80% of CADR (2,6) and are not the object of this chapter.

Unpredictable, idiosyncratic CADR, classified as type B, namely, drug "rashes" or "drug eruptions," are those mostly dependent on immune hypersensitivity reactions. Their clinical presentation and time course is very heterogenous and their recognition is not always easy. Any drug can induce a CADR, each drug can induce several clinical reaction patterns, occurring immediately upon exposure or with a delay of hours, days, weeks, or even months, and there is no universal test to confirm drug hypersensitivity. Some clinical patterns are very typical of a CADR, as toxic epidermal necrolysis (TEN), Stevens–Johnson syndrome (SJS), acute generalized exanthematous pustulosis (AGEP), or fixed drug eruption (FDE). But CADRs occur under a multitude of clinical presentations and at variable severity, often mimic skin diseases that are not usually drug induced (pemphigus, bullous pemphigoid, lupus erythematosus, psoriasis, and lichen planus) or present as maculopapular exanthemas (MPEs) or urticaria that may have other etiology (1,7). Also, it is often difficult to distinguish the relative contribution of the drug or other concurrent causes for the final reaction, as in MPE of infectious mononucleosis induced by amoxicillin or in carbamazepine-induced drug reaction with eosinophilia and systemic symptoms (DRESS) with concomitant HHV-6 reactivation.

Pathomechanisms in drug eruptions include a complex interplay of immune and inflammatory effectors and different targets on the skin and, eventually, other organs. The full process from sensitization to the final clinical reaction is not completely understood, but many progresses have been achieved in the last decades with important implications for our daily practice, both in the management of the reaction (diagnosis, treatment options, complementary tests to confirm the culprit drug), pre- and postpreventive measures and, eventually, also to understand pathomechanisms involved in related dermatosis.

IMMUNE PATHOMECHANISMS IN CUTANEOUS DRUG HYPERSENSITIVITY

Idiosyncratic, unexpected CADRs are immune-inflammatory reactions, mostly representing an acquired and specific hypersensitivity reaction that requires previous sensitization. Nevertheless, in some cases, it is mostly a pseudoallergic reaction or the result of a complex interplay between viral reactivation and drug immune stimulation or a of drug-induced immune receptor activation.

Actually and due to the recognition of susceptibility factors for some drugs some are no more unexpected, namely, abacavir or carbamazepine hypersensitivity, respectively, in HLA-B*5701 for and HLA-B1502, and they can be preventable (8,9).

Hypersensitivity mechanisms in CADR can include any of the four classical mechanisms of immune hypersensitivity defined by Gell and Coombs. They do not participate in an exclusive way, but main mechanisms can be identified in some types of CADR. Type I, or IgE-mediated immediate hypersensitivity, where IgE-laden mast cells or basophils recognize the drug combined with a peptide, is mainly involved in urticaria, angioedema, and anaphylaxis. Drug-specific complement fixing antibodies (IgG or, rarely, IgM) recognizing the drug in a cell surface or as a soluble antigen induce, respectively, a type II hypersensitivity reaction or antibody-mediated cytotoxicity, as in drug-induced hemolytic anemia or thrombocytopenia, or a type III hypersensitivity reaction with excessive formation of immune complexes, complement activation (C3a/C5a), and aggression of small vessels walls, inducing leukocytoclastic vasculitis (6,10). Delayed type IV hypersensitivity, involving T cells that specifically recognize the drug has been documented in allergic contact dermatitis (ACD), MPE, DRESS, or drug-induced hypersensitivity syndrome (DIHS), AGEP, FDE, and in the more widespread and severe SJS/TEN (7,11). For each of these different clinical patterns, delayed hypersensitivity is involved through different subsets of T cells and soluble effectors that recruit a wide range of other cells and orchestrate the final inflammatory response (11).

In some cases the drug modifies the immune response promoting autoimmunity or induces the production of pathogenic antibodies directed against skin structures, as in vancomycin-induced linear

IgA dermatitis, (12) drug-induced pemphigus (13) or terbinafine or esomeprazol-induced subacute lupus erythematosus (14,15).

For a better clinical and pathophysiologic understanding of the main immune-mediated drug eruptions they will be divided into immediate reactions, which include urticaria, angioedema, and anaphylaxis that develop within minutes and up to 6 hours of drug intake and nonimmediate or delayed drug eruptions that begin usually within days or weeks after drug intake or, more exceptionally in previously sensitized patients, within a few hours after drug intake. These include a wide spectrum of clinical patterns, particularly the generalized symmetrical maculopapular or more urticarial exanthema, with or without targetoid lesions, bullae, or pustules, with or without systemic symptoms, which represent the large majority of drug eruptions and which is the main objective of this chapter.

IMMEDIATE ADVERSE DRUG REACTIONS

Immediate reactions occur within minutes to a few hours after drug exposure and present clinically as pruritus, urticaria, and/or angioedema regressing with no residual lesions within minutes to hours. In severe cases, anaphylaxis with systemic symptoms in more than one organ (nausea, abdominal cramps, sneezing, bronchospasm, and dyspnea) can progress to hypotension and shock in its most severe and life-threatening expression—anaphylactic shock (16).

Any drug can induce an immediate adverse reaction. The most severe ones occur with betalactam antibiotics (pencillin G, aminopenicillins, and cephalosporins), iodinated radiocontrast media and muscle relaxants used in anesthesia, whereas the more frequent, although usually less severe immediate reactions, occur with aspirin and other NSAIDs, codein, vancomycin, angiotensin-converting enzyme inhibitors (ACEi), heparins, and insulin (Fig. 9.1) (6,17,18).

In immediate immune-mediated reactions, the drug is coupled with proteins establishing stable covalent bonds with several aminoacids and forms a haptenated protein, which is specifically recognized by IgE fixed on tissue mast cells and circulating basophils. When these hapten–carrier conjugates bridge two IgEs on the cell surface, an intracellular cascade of events induces the liberation of preformed inflammatory mediators from their granules (histamine, tryptase, heparin, cytokines, and chemokines) and produce secondary vasoactive mediators (prostaglandins, leukotrienes, PAF/platelet activation factor), which together are responsible for the vasodilatation, increased vascular permeability, and pruritus observed in urticaria and angioedema. These mediators are also responsible for the systemic symptoms in anaphylaxis (10,19).

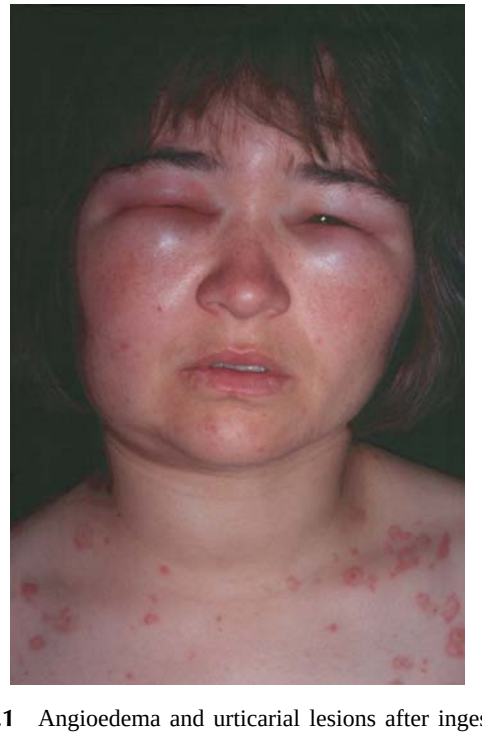


FIGURE 9.1 Angioedema and urticarial lesions after ingestion of an NSAID (diclofenac).

In vitro and in vivo tests can confirm immediate hypersensitivity reactions. In vitro tests demonstrate the presence of a drug-specific IgE by immunoassays (RAST, ELISA, or Immunocapture-fluorenzymeimmunoassay (CAP–FEIA) or drug-specific basophil activation tests (BAT), which measure either increased surface CD63 expression by flow cytometry or mediator release (19–21), but these tests are not randomly available and are not standardized for less frequently responsible drugs. Immediate skin testing (prick or intradermal) and, eventually drug re-challenge, which is not advised in severe cases, can also confirm this diagnosis. These different tests for immediate hypersensitivity (HS) are not always concordant and their specificity and sensitivity vary depending on the drug, test methods used, and experience of the users. If associated they can confirm a diagnosis in a high proportion of patients (>80%) (22).

Immediate drug reactions presenting with urticaria/angioedema, occasionally also systemic but usually less severe symptoms, occur without the identification of a drug-specific antibody (IgE or IgG), and are therefore called pseudoallergic or anaphylactoid (18,22). In these cases tissue mast cells and blood basophils liberate the content of their granules by nonspecific mechanisms, such as the activation of cell receptors for anaphylotoxins (C3a and C5a), direct effect on cellular membrane or in intracellular pathways that regulate degranulation, or imbalance between prostagladins and leukotrienes due to cyclo-oxygenase inhibition by NSAID. Isolated angioedema can occur from the increase of kinins due to ACEi, which inhibit their degradation (18,23,24). In these patients, disturbances in complement and kinin metabolism, namely, in carboxypeptidase that degrades bradykinin, may favor ACEi-induced angioedema, and polymorphisms in the gene for LTC4 synthase may justify familial aggregation of aspirin-induced urticaria (24).

The division of immediate reactions into allergic and pseudoallergic is often artificial. Several drugs that typically induce IgE-mediated reactions, such as muscle relaxants, iodinated radiocontrast media, and heparins, also induce a direct and nonspecific basophil or mast cell activation, which can be responsible for nonspecific positive skin and BAT (20,23). Even for penicillin and fluorquinolones a nonspecific capacity for mast cell activation (albeit low) has been documented in vitro (25). Also, occasionally, drug-specific IgE has been documented in aspirin-induced urticaria and/or asthma, classically considered pseudoallergic (26). Therefore, this makes the distinction between allergic and pseudoallergic reactions difficult, both on clinical and laboratory grounds.

Drug-specific IgG or IgM antibodies can also be responsible for immediate symptoms, because these antibodies give rise to circulating immune complexes and complement activation and induce urticaria with systemic symptoms within the context of serum sickness disease (fever, arthralgia or arthritis, abdominal pain and urticaria, urticaria vascultis, or leukocytoclastic vasculitis), which occurs

either immediately or within a few days of drug administration (6,18). Apart from heterologous proteins, originally responsible for serum sickness, now this is most frequently induced by non-protein drugs, particularly cefaclor, cotrimoxazol, NSAIDs, and diuretics (10).

NONIMMEDIATE/DELAYED DRUG REACTIONS: CLINICAL AND HISTOLOGIC PATTERNS

The main clinical patterns of nonimmediate drug eruptions include maculopapular exanthema, with systemic symptoms in DRESS or DIHS, with pustules in AGEP or with extensive epidermal necrolysis and bullae in SJS/TEN or localized in FDE.

At present, clinical and experimental data confirm the involvement of delayed-type hypersensitivity reactions with the participation of drug-specific T cells in these CADRs: (1,7,10).

1. They usually begin within 7–21 days in the 1st episode and 1–2 days after drug reintroduction (6);
2. Drug-specific positive oral re-challenge with lower doses is usually observed (27);
3. On histopathology there is mainly a dermoepidermal infiltration of activated T cells (28);
4. In a high percentage of cases the culprit drug induces specific positive patch, prick, or intradermal skin tests with delayed readings (29–32); and these tests have an histologic pattern simulating the drug eruption (33–35);
5. In vitro tests show drug-specific T-lymphocyte activation/proliferation or production of cytokines (21,36);
6. Drug-specific T cell lines and clones are isolated from the blood and skin during the acute episode or, later, from positive patch tests (37,38).

Nevertheless, these drug-specific T cells show different in vitro effector activity, in which concerns cytotoxic potential induced by Fas-L, perforin/granzyme B or granulysin, or their cytokine profile (INF-γ, IL2, IL-5, IL-17), which in part is responsible for different phenotypes of the skin reactions (39,40).

Maculopapular Exanthema

MPE, the most frequent pattern of CADR, appears as a generalized symmetric eruption of isolated and confluent erythematous macules and/or papules, often starting in the trunk and spreading to the extremities. Mucosae are not affected and systemic symptoms are mild (malaise and low-grade fever). The reaction develops within 7–14 days after drug intake (within 1 or 2 days in sensitized patients), mainly due to antibiotics (aminopenicillins, cefalosporins, and sulfonamides), allopurinol, and anticonvulsants. The reaction may be mild and regress within a few days, but most often progresses for a few days after drug suspension and fades progressively within 10–15 days, often with desquamation (Fig. 9.2) (7,10,41).

On histology, early lesions show an interface dermatitis with vacuolar degeneration of basal keratinocytes, mild spongiosis, scattered apoptotic keratinocytes, lymphocytes mainly at the dermoepidermal junction, and in papillary dermis with eosinophils along dermal vessels. Lymphocytes are skin homing highly activated T cells, expressing adhesion molecules, mostly CD4+ T cells but CD8+ T cells are also found, mainly in the epidermis (10,28,41). Both T cells, but particularly CD8+, express high levels of perforin, granzyme B, and granulysin (42).

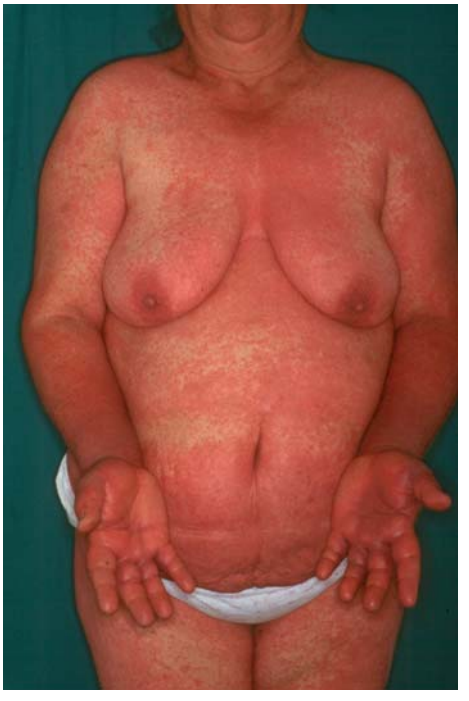


FIGURE 9.2 Maculopapular exanthema from carbamazepine.

Drug-Induced Hypersensitivity Syndrome/Drug Reaction with Eosinophilia and Systemic Symptoms

DIHS/DRESS is a severe life-threatening CADR with diagnostic criteria defined initially by Bocquet et al. and recently and independently revised by a Japanese Consensus Group and the European Group REGISCAR (43,44). A severe maculopapular rash or a generalized exfoliative dermatitis, often with severe facial edema with vesicles or pustules (Fig. 9.3) occurs in most cases. Systemic symptoms (fever, malaise) and internal organ involvement affects mainly the liver (severe hepatitis and cholestasis), lymph nodes (tender lymphadenopathy), kidney, heart (myocarditis), and/or lung. Leukocytosis with circulating atypical (activated) lymphocytes and eosinophilia (>700/μL or 10%) are frequently observed, with eosinophil counts increasing late during the course of DRESS (43,45).

In some patients the skin reaction in DRESS may be clinically similar to a MPE and only the association of systemic symptoms and their severity may allow the diagnosis of DRESS. Actually, there seems to be a continuum between MPE and DRESS, with some patients with MPE from carbamazepine or allopurinol having a low-grade fever and slight hepatic cytolysis that regress more rapidly, and therefore do not fulfill the definitive criteria for DRESS.

DRESS has a delayed onset, more than two weeks up to 6–8 weeks after the initiation of the drug, usually an anticonvulsant, allopurinol, sulfasalazine, abacavir, nevirapine, dapsone, or minocycline. It also regresses slowly often with exacerbations, either related with steroid withdrawal, viral reactivation, or administration of a related or nonrelated drug (1,46,47).

Actually this particular type of drug-induced reaction seems to be highly dependent on herpes virus infection or reactivation, particularly HHV-6 or 7 or EBV, detected by an increase in IgG titer or viral DNA by polymerase chain reaction, and this has even been added by the Japanese team as one criterion for the diagnosis of DRESS (43). Nevertheless, positive patch tests with a very low concentration (1%) can be observed later, particularly when carbamazepine or abacavir is the culprit drug (45,48).

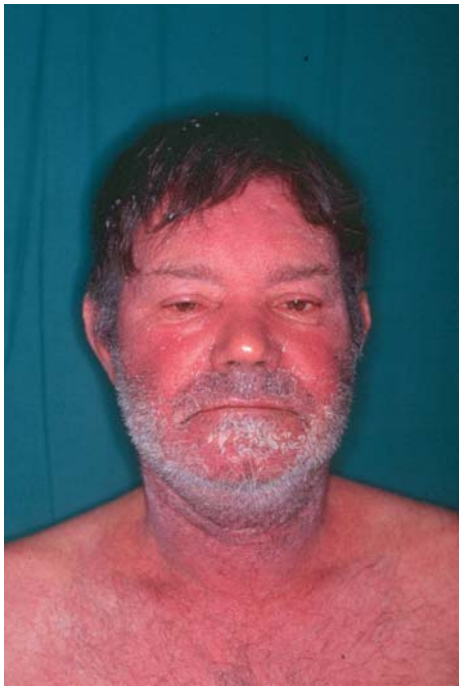


FIGURE 9.3 Exfoliative dermatitis with facial edema in a case of drug reaction with eosinophilia and systemic symptoms induced by allopurinol.

In DRESS, CD4+ and CD8+ T cells infiltrate the dermis and epidermis (49) with a lichenoid pattern or often with a dense dermoepidermal infiltrate mimicking mycosis fungoides. This lymphomatous infiltrate in the skin and in lymph nodes has suggested a previous designation of drug-induced pseudolymphoma (50). Eosinophils can infiltrate the skin, particularly in perivascular dermis, and epidermal changes, such as basal cell vacuolization and scattered apoptotic keratinocytes, are also observed, as in MPE.

Acute Generalized Exanthematous Pustulosis

AGEP is a very peculiar reaction pattern induced by drugs in more then 90% of cases, mainly by aminopenicillins and other antibiotics, diltiazem and terbinafine (7). It is characterized by the acute onset of symmetrical widespread edematous erythema covered by small nonfollicular sterile pustules, predominating in the face and body folds, high fever (>38°C), leukocytosis, neutrophilia and, occasionally, eosinophilia (Fig. 9.4A, B). The reaction develops within one week of drug intake and regresses in 5–10 days after drug withdrawal.

Early biopsies from AGEP show a dermoepidermal infiltration of T cells, mainly CD4+DR+CD25+, with discrete vacuolar keratinocyte degeneration and a perivascular infiltrate of lympocytes and eosinophils, sometimes with vasculitis (51,52). Lesions progress to spongiotic vesicles that soon transform into epidermal and subcorneal pustules due to neutrophil accumulation (52,53). This same pustular pattern with spongiform neutrophilic subcorneal pustules also occurs at positive patch tests, after 72 hours, which make them a very useful tool to study the pathomechanisms involved in AGEP (7,33,34,54).

Stevens–Johnson Syndrome/Toxic Epidermal Necrolysis

SJS and its more extensive variant, TEN, represent a life-threatening pattern of CADR characterized by widespread symmetrically distributed macular lesions, showing typical or mainly atypical targets, with central bulla, that coalesce to form large sheets of necrotic epidermis covering more than 30% of the body surface area in TEN and 1–10% in SJS (Fig. 9.5). To include the whole spectrum from SJS, overlap SJS/TEN and TEN, Roujeau et al. recently suggested the designation of exanthematic necrolysis (55).

The eruption is often preceded by fever, malaise, painful mucosal erosions and, as the skin rash progresses from the head to the extremities, fever and systemic symptoms occur in a variable intensity and combination. Conjunctivae, oral and genital epithelial shedding is usually intense and painful, and can be associated with epithelial necrosis of the oropharynx, gastrointestinal tract, trachea, and bronchia. SJS/TEN are due to drugs in more than 90% of cases, usually an antibiotic (sulfonamide), allopurinol, an anticonvulsant (lamotrigine, carbamazepine), nevirapine, or an NSAID (oxicam) (13).

There is a variable degree of skin inflammatory infiltrate, ranging from almost absent to a dense dermal T infiltrate, which seems to correlate positively with the percentage of skin detachment and, consequently, with the mortality rate (56,57). Factor XIIIa+ dermal dendritic cells (DCs) are increased contrasting with a reduction of CD1a+ Langerhans cells, CD4+ and CD8+ T cells are scattered in the dermis and many lymphocytes are found in the blister fluid, mostly activated cytotoxic CD8+ CD56+ T cells and NK T cells rich in granulysin (55,58,59). But the most striking histologic marker of TEN is the keratinocyte cell death (apoptosis) extending to all epidermal layers.

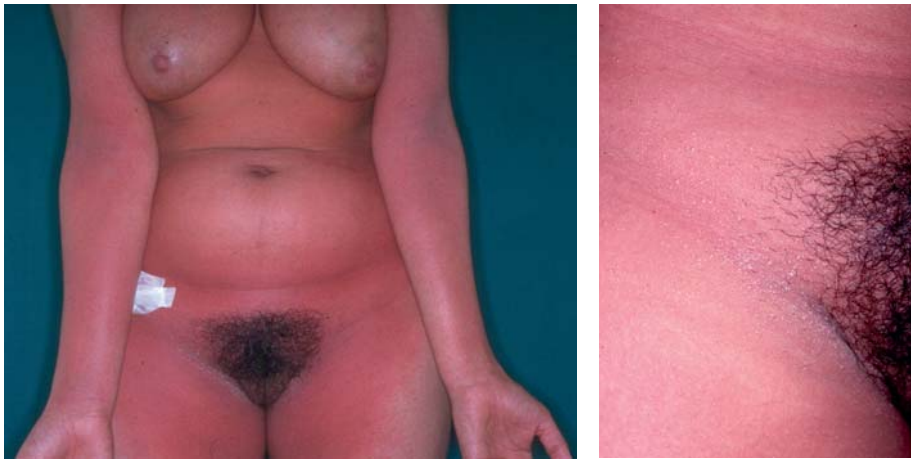
Fixed Drug Eruption

FDE is a particular drug hypersensitivity reaction involving the skin and mucosa. It occurs as round erythematous macules or plaques, some with bulla and epidermal detachment, which regress spontaneously within 10–15 days with a gray-brown hyperpigmentation (Fig. 9.6). Lesions recur on the same place within hours after drug reintroduction, often with new lesions. The number and size of the lesions may vary from a few small to large, coalescent lesions with a widespread involvement, which may be difficult to distinguish from TEN, although it resolves rapidly with no systemic symptoms (13).

At the acute phase there is a mononuclear inflammatory infiltrate, mainly at the dermoepidermal junction, with hydropic degeneration of basal keratinocytes and scattered or more extensive keratinocyte apoptosis, eventually involving the whole epidermal thickness, as in TEN. Upon regression, melanophages accumulate in the dermis and CD8+ T cells persist in the epidermis in abnormal numbers for years after clinical resolution (60). They are drug specific and are easily activated upon drug contact, which explains frequent positive epicutaneous tests, only on residual lesions, within a few hours of contact and with histhopathology typical of an FDE (61,62).

NONIMMEDIATE/DELAYED DRUG ERUTPIONS: SENSITIZATION, DRUG RECOGNITION, AND SUSCEPTIBILITY

Delayed CADR from systemic drugs, presenting as maculopapular exanthema with its variable clinical patterns (targets, bullae, or pustules) with or without systemic manifestations, involve complex immune mechanisms that are not completely understood,



(**A**) (**B**)

FIGURE 9.4 (**A**) Acute generalized exanthematous pustulosis induced by amoxycillin. (**B**) Detail of (A) Small pustules mainly on body folds in acute generalized exanthematous pustulosis.

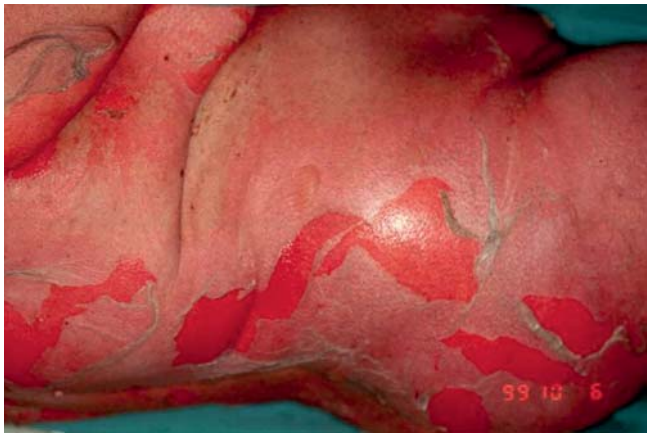


FIGURE 9.5 Extensive skin detachment involving about 60% of the body surface area in a patient with toxic epidermal necrolysis from allopurinol.

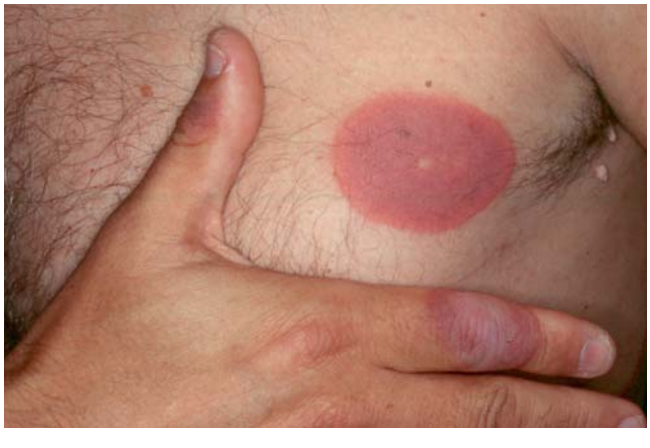


FIGURE 9.6 Typical round erythematoviolaceous lesions in fixed drug eruption from piroxicam.

also because there is no universal animal model to study CADR (39). In which concerns delayed reactions to epicutaneous drug application, presenting as ACD, immune mechanisms involved, including the sensitization and elicitation phases, have been recognized with increasing details both in man and animals.

Topical drugs, as haptens, combine with small peptides and promote an innate inflammatory immune response in resident skin cells (IL-18, IL-β, TNF-α), which recruits other inflammatory cells. Resident Langerhans cells and other DCs, stimulated directly by the hapten and by the epidermal cytokines/chemokines, mature, and migrate from the epidermis to the regional lymph nodes carrying the processed haptenated proteins bound to the HLA molecules. In the lymph node, naïve T cells that recognize the antigen in an adequate ambient of co-stimulatory molecules develop into sensitized CD8+ and CD4+ specific effectors and memory T cells expressing CLA and CCR4+, mostly with a Th1 or Th17 pattern of cytokine production. These cells recirculate and are recruited to the skin in a further drug application, initially by the skin innate cytokines and chemokines IL-1β, TNF-α, CCL2, CCL5, CCL20, and CCL22 and, after the influx and activation of the specific T cells and their production of IFN-γ, IL-1β, and IL-17, also by the secondary chemokines CXCL10 (IP-10), CXCL9 (Mig), and CXCL8, which increase the inflammatory response. Drug-specific effector T cells have a cytotoxic effect on keratinocytes and Langerhans cells carrying the antigen, whereas the infiltration of regulatory T cells (Treg) may dampen the inflammatory response of ACD (63). On histology there is then a dermoepidermal T-cell infiltrate, with lymphocyte exocytosis, spongiosis, and vesiculation with scattered apoptotic keratinocytes and clinically an erythematopapular or vesicular pruritic reaction.

ACD from topical drugs can become widespread, sometimes simulating a systemic drug eruption, due to percutaneous drug absorption. Also, systemic exposure to the same or similar drugs can induce systemic contact dermatitis, presenting as a morbiliforme-like eruption or as a “baboon syndrome” or as symmetrical drug-related intertriginous and flexural exanthema or an acrovesicular dermatitis (64–66).

Therefore, similar mechanisms may be involved in the delayed reactions to topical and systemic drugs, but with many particular aspects that distinguish their pathomechanism from ACD, namely, in which concerns the responsible hapten/antigen for the specific drug recognition, the process of antigen presentation and cells involved in this activity, drug recognition by the immune system, including a possible direct pharmacologic drug effect, concomitant factors that influence drug sensitization and/or elicitation of CADR, genetic susceptibility markers (HLA haplotypes), which

are dealt with in the following sections. As in contact dermatitis, the capacity of the drug to stimulate the innate skin immune system may be an important step in awakening the immune system to develop the adaptive immune response (67). Interestingly, even the capacity of the drug to induce the primary innate immune response is exclusive to individuals who develop the hypersensitivity reaction; DCs from controls stimulated in vitro do not show the same maturation patterns (68), therefore suggesting interindividual variation in the innate response.

Antigens and Haptens in Drug Hypersensitivity

Drugs are not usually directly recognized by the immune system and do not induce a direct specific immune response, which means they are not antigenic or immunogenic on their own. They usually function as a hapten that needs combining with proteins/peptides to become antigenic and capable of inducing an immune response.

Also, often the drug is a prohapten in this process: the drug itself is not recognized by the immune system, but another chemical formed as an intermediate or final product of drug metabolism or bioactivation (69). Main drug metabolization is usually accomplished by hepatocytes but DCs also have rich enzymatic machinery capable of producing the intermediate or bioactive drug metabolites, as shown for sulfametoxazole (70,71) and abacavir (72).

These reactive drugs, oxidative intermediates, or final metabolites can establish covalent bonds with nucleophilic aminoacids (mainly cysteine or lysine) in soluble or cellular peptides (70). This has been studied for penicillin which, after opening the beta-lactam ring, binds covalently to a thiol group to form the penicilloyl determinant and for sulfametoxazole whose metabolite nitroso-sulfametoxazole binds covalently to proteins (73–75). Abacavir is processed intracellularly and binds exclusively to a few aminoacids in the antigenic grove of HLA-B*5701 forming the antigen that is recognized by the T-cell receptor (TCR). A single aminoacid replacement in this groove is enough to prevent this association (72).

Therefore, the immune system can specifically recognize the drug and/or a metabolite bound to proteins/peptides or, eventually, a self-protein (e.g., HLA molecule) modified by the reactive drugs or metabolites.

Moreover, some drugs stimulate the innate immune system in a nonspecific way, acting as a "danger signal," which may be important to trigger the specific immune response. Some drugs are used therapeutically to obtain such an effect, such as imiquimod that directly activates TLR-7 in DCs to enhance the presentation of viral or tumor antigens. It is increasingly recognized, at least in vitro, that drugs inducing delayed drug eruptions, such as carbamazepine, amoxicillin, sulfametoxazole, and abacavir, activate directly DCs as occurs with contact allergens (68,72,70,76). They activate intracellular signaling pathways and induce phenotypic changes in DC in vitro, with expression of markers of DC maturation (CD40 and/or CD86) and produce co-stimulatory cytokines (IL-1β, IL-18) and chemokines, which enhance antigen presentation and T-cell activation.

Drugs that induce photosensitivity need a previous photoactivation, where UV energy is used to transform the drug into a reactive metabolite that combines with proteins to form a hapten recognized by T cells, as shown for ketoprofen (77), triflusal (78), or piroxicam. This NSAID is degraded by UVA into a photoproduct chemically and antigenically similar to the thiosalicylate moiety of thiomersal and, both after topical or systemic exposure, induces photoallergy in individuals with contact allergy to thiomersal (79,80).

Antigenic Presentation in Drug Hypersensitivity

Professional or recruited DC rich in HLA and co-stimulatory molecules are necessary for drug presentation to the immune system. For some drugs antigen processing is necessary before the combination with HLA molecules, either HLA classes I and II. Nevertheless, some haptenated peptides can bind directly to the HLA molecule without previous processing or the drug can simply establish transitory binding with peptides within in the HLA groove, as shown with carbamazepine, lamotrigine, and several fluorquinolones (73,81). Some drugs have a long-lasting binding with DC and, at least in vitro, they can be detected in culture for many days (71), which may explain the progression of the skin reaction for a few days after drug withdrawal: it is possible that in vivo antigen presentation will proceed for days and if, the drug is also firmly bound to target cells, they may be a long-lasting target for effector cells.

Skin DCs, both Langerhans cells and other epidermal or dermal DCs, are responsible for antigen processing and presentation after epicutaneous drug application. For systemic drugs tissue or circulating DC, peripheral blood mononuclear cells (PBMC) or, eventually, skin DC may participate in antigen presentation and sensitization of naïve T cells, but at present, no data has shown which DCs participate in antigen presentation for nonimmediate CADR, neither in which lymph nodes the cross-talk between DCs and T cells occurs to generate memory cells and effectors, particularly CLA+ T cells with skin-homing capacity.

Nevertheless, human DCs developed in vitro from PBMC of healthy volunteers and patients with sulfametoxazole-induced MPE can be directly stimulated by the drug, (70) and blood-derived immature DCs, particularly from amoxicillin-reactive patients, increase the surface CD86 and DR expression in the presence of the drug in vitro and, in parallel, increase the capacity to stimulate T-cell proliferation (68).

The subtype of DCs usually involved in viral recognition—plasmocitoid DCs (pDC)—may also have an important participation in drug antigen presentation or may be involved in the proliferation of virus-related T cells observed in many drug eruptions, particularly in DRESS (67). B cells and NKT cells may also participate in the presentation and/or activation of effector T cells, but little is known about their participation in nonimmediate skin reactions. Contrary to DC, circulating B cells and monocytes isolated from patients with delayed reactions from amoxicillin failed to activate T cells from these patients in vitro (68).

Drug Recognition by the Immune System

Drugs are specifically recognized by antibodies, IgE in mast cells and basophils, IgG or IgM fixed on cells or in soluble immune complexes, in immediate hypersensitivity or vasculitis, but there is no documentation of antibodies recognizing the drug in delayed CADR. Drugs are mainly recognized by T cells, both CD4+ and CD8+, through their specific receptor, the TCR, mostly in an Major histocompatibility complex (MHC) restricted manner, by class I for CD8+ and class II for CD4+ T cells, as shown for penicillins, cephalosporins, norfloxacine, sulfamethoxazole, carbamazepine, lamotrigine, and phenobarbital (69).

HLA class I or class II molecules carry the drug bound covalently or noncovalently to a peptide or to particular aminoacids in the HLA groove (74), and the complex is recognized, as for classical antigens, by αβ TCRs with the relevant sequence of aminoacids in its

hypervariable region. Abacavir tightly combined with HLA-B*5701 is recognized by TCRs with no predilection for any Vβ region (72). Carbamazepine combined with HLA-B*1502 is recognized by T cells with the Vβ-11 region with the ISGSY sequence in TCR and induces an oligoclonal proliferation, but this has been shown only for SJS/TEN, not other drug eruptions (9,55). Other carbamazepine and lamotrigine-reactive T-cell clones generated from infiltrating skin and circulating cells, which recognize the drug on HLA class II–matched antigen presenting cells, harbor almost all Vβ 5.1 chain, suggesting a superantigen-like effect (82).

The perfect match between specific HLA antigen presenting cells laden with the drug in the context of a mature DCs with adequate co-stimulatory molecules and in the adequate ambience of cytokines, and the TCR activate the clone, or the few clones, that harbor the specific TCR and sensitize naïve T cells or induce proliferation of memory effector T cells.

For each drug inducing a CADR, there is not a unique way for T-cell recognition. For instance, some T-cell clones or T-cell lines isolated from patients with cotrimoxazole-induced MPE, in vitro, recognize sulfamethoxazole, whereas others recognize intermediate metabolites, such as hydroxylamine sulfamethoxazole or nitroso-sulfamethoxazole, presented directly in the HLA groove or after antigen processing and with MHC restriction (59,73,74). The type of recognition dictates the pattern of cross-reactivity: T cells recognize sulfametoxazole and other anti-infectious sulfonamides with a same conformational structure (59), whereas clones that recognize nitroso-sulfametoxazol have a more restricted cross-reactive pattern (74).

Like in this example, it is important to know how drugs are recognized and particularly which drug moiety is responsible for the immune recognition as this may be determinant in understanding cross-reactions. Although not studied at T-cell level, the antigenic moiety of piroxicam recognized by the immune system, which depends on the pattern of drug eruption, also dictates the cross-reaction pattern. The thiosalicylate moiety, which formed after UVA radiation, is responsible for photoallergy. As this photoproduct is exclusive for piroxicam, other oxicams, such as tenoxicam, can be safely used in photoallergy. On the other hand, in FDE the immune system recognizes another oxicam moiety, which is common to tenoxicam and, therefore, all patients with FDE to piroxicam cross-react with tenoxicam (80,83,84).

Pharmacologic Drug Effect: The p-i Concept

Another possible mechanism of drug interaction with the immune system has been proposed by Werner Pichler, the "**p**harmacological interaction with **i**mmune receptors," the p-i concept (73). As for other pharmacologic receptors the drug can by its steric and electronic features establish reversible interactions with immune receptors in cells with other antigen specificity and, in certain conditions, promote their activation and expansion to generate the skin reaction (40,85).

This is based on the fact that some drugs, such as X-ray contrast media, induce T-cell mediated skin eruptions on the first contact and usually within a few hours, therefore with no time for the sensitization process (81). Also some drugs do not establish long-lasting covalent bounds with MHC in antigen presenting cells, in vitro some T-cell clones are activated with no need for an active antigen processing/presenting cell, activation is easily hindered by simply washing the drug from the cells, many T-cell clones exhibit no MHC restriction and there are more T-cell clones activated by the drug then those that exhibit the specific TCR (85). Therefore, there is the possibility that some drugs transiently bind proteins of the MHC complex or particularly the TCR, even outside the antigenic groove, and maintain enough contact between the MHC of the antigen presenting cell and the TCR of memory T cells to induce their activation and expansion responsible for the reaction. The drug would act like a superantigen, although there is no definitive identification of particular TCR Vβ regions associated with drug eruptions, except for a few carbamazepine and lamotrigine clones that have a predominant αβTCR with the Vβ 5.1 chain (74,82).

A previous state of T-cell activation, such as a viral disease (EBV, HHV-6, HIV disease) or concomitant immune-inflammatory disease, might enhance the T-cell response as these cells have a lower threshold for activation (73,81,85). This also explains the exclusive activation of T cells in this reaction models, sometimes escaping the involvement of the innate immune response (40).

Although there are many arguments that support this theory it has not been fully demonstrated experimentally and it is important to reinforce that this effect does not exclude the drug activating the immune system as a hapten/prohapten (85).

Concomitant Predisposing Factors

According to the p-i concept and also the hapten hypothesis, the immune status of the patient during drug exposure is often important for the development of the CADR. Concomitant aggressions (exposure to other reactive chemicals or drugs) and chronic immune-inflammatory diseases (Still's disease, systemic lupus erythematosus) that promote cell death with exposure of endogenous peptides (death-associated molecular patterns) or infectious disease with bacterial toxins, viral proteins, or nucleic acids (pathogen-associated molecular patterns) that stimulate Toll-like receptors in keratinocytes, other epithelial cells, DCs, and other antigen presenting cells, act as "danger" signals that alert the adaptive immune system that goes into a preactivated state: monocyte/macrophages or DCs become increasingly capable of presenting the drug to T cells (67,86). According to the p-i concept, the preactivation of a large population of T cells involved in the previous immune-inflammatory process, therefore with a reduced threshold for activation, may also enhance T-cell proliferation induced by the drug (81).

These concurrent factors are of extreme importance, both during active drug sensitization and the development of the CADR, as shown in several situations: concomitant use of aminopenicillins and allopurinol seem to represent a risk factor for developing CADR (87); cystic fibrosis patients either due to perturbed immune status with frequent infections or extensive exposure to antibiotics have a much higher risk of drug reactions to antibiotics (40); systemic lupus erythematosus or HIV-infected patients are more susceptible to CADR, particularly from sulfonamides (13,74); during Epstein–Barr virus (EBV) or cytomegalovirus (CMV) infection, antibiotics, particularly aminopenicillins, induce MPE in a high proportion of patients but only a few become sensitized and develop a skin rash on drug re-exposure without the concomitant infection (88); and during the last decades, attention has been drawn to the association of DIHS/DRESS with human herpes virus type 6 (HHV-6) primoinfection or reactivation (43,46).

Nevertheless, the relationship between viral reactivation and the drug may be the inverse as recently it has been shown that T-cell activation by the drug may enhance viral replication, as shown for amoxicillin in EBV infection and herpes virus after carbamazepine T-cell activation (89).

For photoactive drugs their inherent phototoxic potential, with the capacity to generate reactive oxygen species (ROS) and other molecular and cellular changes (namely in DNA and cell membranes) may be important to initiate an innate immune response that will favor sensitization to a drug photoproduct. Actually most drugs that develop photoallergy also have some inherent phototoxic potential (90).

Genetic Predisposing Factors

Many genetic factors in the metabolizing pathways, immune-inflammatory genes, TCR repertoire and, particularly, certain HLA haplotypes predispose individuals to drug eruptions induced by some drugs.

In drug detoxification process polymorphisms within drug metabolizing enzyme genes, namely, in cytochrome P450, can give rise to different intermediate reactive (or nonreactive) drug metabolites or to distinct amounts of the culprit metabolite (91).

Polymorphisms in immune-inflammatory response pathways may increase the risk of some particular drug reactions: predisposition to produce higher levels of soluble Fas ligand and polymorphisms in the TNF-promoter region may correlate with an increased severity of drug reactions (11,92).

Some HLA haplotypes, which may be related to the capacity of the drug to combine/or insert into HLA groove of antigen presenting or target cells, have been related with increased/or reduced capacity to develop a drug eruption to a certain drug (5). The first strong association was shown, in 2004, for HLA-B*1502 in carbamazepine-induced SJS/TEN in Han Chinese in Taiwan (93,94). Later, other Asian populations, except the Japanese, were shown to have this association, only for SJS/TEN from carbamazepine and other antiepileptics such as oxcarbazepine, phenytoin, and lamotrigine (95), which often induce adverse reactions in carbamazepine-sensitive patients. This association is not extensive to Europeans where a more recent study found association between HLA-B*3101 and several patterns of carbamazepine-induced CADR, namely, DIHS/DRESS, SJS/TEN, and MPE (96), both in Europeans and Japanese (95). A strong association was also detected in Asia for HLA-B*5801 and severe drug reactions from allopurinol, both SJS/TEN and DIHS/DRESS, and this association is also confirmed for Europeans (95,9), including our experience where 50% of patients with severe CADR from allopurinol showed HLA-B*5801, compared with 1.96% in controls (personal experience).

One of the most strong and established association worldwide is for HLA-B*5701 and a potentially fatal hypersensitivity syndrome from abacavir, with some particular characteristics: it develops in a short time interval, with no eosinophilia but a significant hepatic and gut involvement and is confirmed by patch tests (35,48,97,98). The positive predictive value is 55% and the absence of this haplotype has a negative predictive value near 100% (9,98). This makes the search for this haplotype mandatory before initiating therapy. Interestingly, an isolated study from Italy, dating back from 1997, had already shown a strong association between FDE from feprazone and HLA-B22 (99).

In all these studies the stronger associations occurred with HLA-B, therefore class I, justifying the frequency of drug-specific CD8+ T-cell clones involved in cases of drug hypersensitivity, namely, for abacavir and allopurinol (95,98) On the other hand, for nevirapine, which induces a more heterogenous pattern of hypersensitivity reactions, apparently dependent on drug-specific CD4+ T cells and dependent of patient CD4+ cell count, the strongest but not definite association was found with the class II antigens, HLA-DRB1*0101 (98).

Which seems more difficult to explain is why some HLA haplotypes predispose to one pattern of drug reaction, whereas others predispose to other patterns and why some individuals do not develop the reaction although they have the proper haplotype. In this setting it is most understandable that concomitant factors are important for developing hypersensitivity.

Apart from HLA susceptibility, other still unknown individual particularities might explain why immature DCs from hypersensitive patients are more responsive in vitro to the drug than controls, as shown for amoxicillin (68).

NONIMMEDIATE/DELAYED CADR EFFECTOR MECHANISMS AND REACTION PATTERNS

The effector phase of the immune drug reaction involves drug-specific T cells, which confers the specificity of this reaction, but most of the aggression is developed by other auxiliary nonspecific cells recruited to the site of inflammation and their soluble products and their interaction with skin resident cells, particularly the keratinocytes.

The innate immune response that develops upon drug exposure and the distinct subsets of T cells with distinct cytokines/chemokines and aggressive machinery that orchestrate the inflammatory skin reaction, are responsible for the different patterns of drug reactions observed. Concerning the predominance of a T-cell subset, a subdivision of delayed hypersensitivity T-cell reactions has been made into type IVa, IVb, IVc and, more recently, type IVd (11). They represent, respectively, the reactions mediated predominantly by T-helper 1 (IFN-γ), T-helper 2 (IL-4 and IL-5), cytotoxic reactions [cytotoxic T lymphocytes (CTL), CD8+ rich in perforin, granzyme B, granulysin, and FasL], and CXCL8 (IL-8) secreting T cells that promote neutrophilic inflammation (100). Nevertheless, this may be an artificial division mainly due to more recent recognition of the importance of drug-specific CD8+ cells in most delayed ADR, with many CD4+ cells participating as regulatory T cells.

Eventually, individual patients' characteristics or concomitant factors occurring during drug exposure will also influence the final clinical outcome. Actually it is not completely explained why for patients exposed to the same drug, some develop an MPE, others an AGEP or some have bulla, either limited in FDE or with extensive skin involvement in SJS or TEN.

Main Effector Cells and Their Mediators

Drug-specific T cells have been isolated from the blood or from the skin, both during the acute reaction or at different time points from positive skin tests and their capacity for specific stimulation has been studied in vitro under different settings (with or without viable antigen presenting cells of different subtypes, other autologous or heterologous cells and in different cytokine environments).

They are memory effector T cells, with different proportions of CD4+ and CD8+ specific cells, also NK and NKT cells, depending on the pattern of CADR and drug. These cells exhibit different functional activity, namely, different cytokine production or different aggressive machinery.

Circulating drug-specific T cells, as evaluated by enzyme-linked immunospot (ELISPOT) assay, represent 30–125 cells/10^6 PBMC both for amoxicillin and sulfametoxazol, similar to the circulating nickel-reactive T cells in patients with ACD from this metal (36).

Usually drug-specific T cells exhibit high levels of cutaneous leukocyte antigen (CLA) and CCR10 turning them highly susceptible for skin migration, and show markers of activation, such as CD25, HLA-DR, and adhesion molecules, such as CD11a-CD18 (LFA-1). Most isolated cells and T-cell clones are type 1 and/or type 2 CD4+ and CD8+ T cells, respectively, with HLA class II or class I restriction (69). Th17 cells, as well as CD4+ T cells with regulatory activity (IL-10 and TGF-β) are also involved (40).

Among drug-specific CD4+ cells production of IFN-γ is usual, often accompanied by IL-4, IL-5, and IL-13. Although INF-γ is the main cytokine produced by these cells, IL-5 (a Th2 cytokine particularly activating eosinophils) is also an important marker of many drug eruptions (37,38), and not only in DRESS where eosinophilia is significant (69). For the ELISPOT assay, which detects in vitro cytokine production by PBMC in the presence of the culprit drug, its sensitivity can be increased if, apart from INF-γ, IL-5 detection is also used (21). Some drug-specific CD4+ cells show also cytotoxicity against keratinocytes, mediated by perforin and granulysin, therefore reinforcing their capacity to cause keratinocyte damage in the skin (37,38,41).

For some drugs, such as abacavir, and particularly in certain types of drug eruptions the main memory effector T cells are drug-specific CD8+ cells with type 1 cytokine patterns. They produce high levels of IFN-γ and contain cytolytic granules rich in perforin, granzyme B, and granulysin, which degranulate upon stimulation and exert high cytotoxic effect (69). These cells induce target cell apoptosis after cell-to-cell contact within the context of HLA molecules, namely, against autologous keratinocytes expressing HLA-class I combined with the culprit drug. This is in agreement with the accumulation of granulysin- and granzyme B-rich cells with the basal cell layers in areas of vacuolar degeneration and particularly in SJS/TEN and FDE where there is a major degree of keratinocyte apoptosis (40,42). Cytolytic granule contents can exert their action also at a distance, without the need for cell-to-cell contact (101,102). Granulysin, particularly the 15 kDa form of this cationic cytolytic protein, significantly increased in the blister fluid of TEN (300× compared with blisters from burn injuries), is liberated into the extracellular fluids, particularly after T-cell activation, and in the high concentrations detected induces apoptosis even without cell-to-cell contact (103).

These cytolytic granules, particularly granulysin, are not exclusive of CD8+ cells and can be secreted also by NK cells and NKT cells. During the acute eruption a subset of NK cells (CD3-, NKp46+) infiltrate the skin, particularly in the CADR with the highest levels of cytotoxicity. In TEN they represent up to 13% of lymphocytes and accumulate in the dermal/epidermal interface and blister fluid, whereas in MPE they are less than 4% and are present in the dermis. Although they do not have a TCR to recognize the drug, in vitro, they are stimulated in a drug-specific manner to upregulate granulysin production (42).

The different contribution of these subtypes of effector T cells and other particularities of the inflammatory response, as shown below, will determine which reaction pattern will develop.

Effector Mechanisms in Maculopapular Exanthema

In MPE exanthema the skin is infiltrated by skin homing CLA+, CD4+, and CD8+ T cells, with CD8+ T cells mainly in the epidermis and CD4+ in the dermis around blood vessels. They are attracted from the blood through the interaction of their adhesion molecules (CLA/LFA-1) with ICAM-1 and E-selectin in endothelial cells and by keratinocyte chemokines, such as the CCL-17/22 and CCL-27 (CTACK-cutaneous T-cell attracting chemokine) that selectively recruit skin homing memory T cells expressing, respectively, the CCR4 and CCR10 receptors (74,28,104,105).

T cells secrete an heterogenous profile of cytokines and chemokines, which promote inflammation: type 1 cytokines (IFN-γ) activate DCs and keratinocytes increasing their expression of HLA molecules, both class I and class II, that bind the drug and present it to T cells or are the target for apoptosis; IL-5, a type 2 cytokine, along with the eotaxin/CCL-11 is responsible for the recruitment and activation of eosinophils, a local and systemic hallmark of cutaneous maculopapular drug eruptions; during the acute phase, both CD4+ and CD8+ T cells expressing perforin, granzyme B and granulysin have cytotoxic activity against keratinocytes, which suffer vacuolar degeneration near the basal layer or apoptosis in scattered cells in the upper epidermal layers (41). Most recent work considers drug-specific CD8+ as the main, or at least, the first effector cell in MPE, as they infiltrate positive patch tests to drugs in the few hours after drug application and before the recruitment of CD4+ cells, which may act more as regulatory T cells (39,63).

Effector Mechanisms in Drug-induced Hypersensitivity Syndrome/DRESS

In DRESS circulating activated CLA+, CCR10+, CCR4+ skin homing T cells, mainly CD8+, and their main cytokines, INF-γ, TNF-α, and IL-5, increase in the blood, in proportion with the severity of skin disease (47,106). CD4+ and CD8+ T cells infiltrate the dermis and epidermis, in some cases with such an exuberant density and activation markers that they can mimic a cutaneous T-cell lymphoma (43,50). Drug-specific T cell clones, both CD4+, CD8+ and CD4+CD8+ were isolated from the blood and skin, particularly for carbamazepine and lamotrigine (74), whereas for abacavir-specific T cells it was CD8+ (48).

T-cell clones are rich in perforin and secrete high amounts of IFN-γ, which seem to control the duration and severity of the inflammatory response (74). IL-5, or eventually IL-17E, secreted by T cells and eotaxin are responsible for eosinophil recruitment and activation (82,107), and eosinophils are supposed to mediate tissue damage in the skin and systemic organs.

Although, as in MPE, drug-specific T cell clones have been isolated in DRESS and patch tests with the drug, namely, with carbamazepine and abacavir are very often positive and specific (45,97), the pathomechanisms involved seem to be complex and not exclusively dependent on the drug.

During the acute phase there is a concomitant reactivation of herpes virus in most cases (>70%), mostly HHV-6, but also HHV-7, EBV, and CMV, detected by anti-HHV-6 IgM, increase in IgG titer

in blood or viral DNA particles in the blood or skin (43,106). There is no definitive explanation for this viral reactivation and its relationship with the skin and systemic injury observed in DRESS.

Some theories suggest the drug first induces immunosuppression, with hypogammaglobulinemia and/or increase of regulatory T cells that suppress the memory T cells that control viral proliferation (43,108), which favors viral reactivation responsible for the systemic symptoms as well as for exanthema and its frequent reactivations (43,46,109). This would explain for instance the long latency period between drug introduction and the first symptoms of DRESS, but no signs of specific viral aggression are detected in DRESS involved tissues, namely, in the skin, and the efficacy of antiviral drugs is relatively low.

According to other explanations, damage in DRESS is due to the immune antiviral response. After an initial immunosuppression, and particularly after drug suspension, the recovery of CD4+ and/or CD8+ cells induces an immune reconstitution inflammatory syndrome with damage of the tissues where the virus/drug is localized, as observed in AIDS after HAART treatment. This might explain negative lymphocyte transformation tests during the acute phase, whereas they become positive by 5–7 weeks, when there is a full immune reconstitution with normalization of the circulating numbers of naïve T regulatory cells (CD4+CD25+^{bright}FoxP3+) with skin homing properties (CLA+CCR4+) found in high amount in the blood during the acute phase (43,108). This recovery might explain the occurrence of enhanced responses to other drugs and autoimmunity that may develop after DRESS (40,43).

Other theories propose that virus-activated T cells, which often recognize several of the herpes virus associated with DRESS, exhibit cross-reactivity with culprit drugs. Therefore, drug exposure induces expansion of both the viral- and drug-specific T cells responsible for the damage (43).

Still, based on the isolation of drug-specific T cell clones, drug-induced T-cell proliferation might reactivate herpes virus harbored in a latent phase in T cells. Actually, recent studies have shown, in vitro, induction of viral proliferation in infected cells in the presence of carbamazepine, a frequent cause of DRESS, but also with sodium valproate (89), the drug we usually use when an anticonvulsant in highly needed in patients with DRESS from aromatic anticonsvulsants (45). Also, using EBV-transformed B cells from DRESS patients, Picard et al. have found an increased number of EBV copies in culture in the presence of carbamazepine, allopurinol, and sulfamethoxazole, reinforcing the fact that drugs induce viral proliferation but only in cells from DRESS patients. Moreover, they found that most CD8+-activated T cells isolated from skin, liver, and lung during DRESS exhibit CDRs with sequences common to anti-EBV T-cell clones, suggesting that DRESS is a multiorgan disease mediated by CD8+ T cells recognizing herpes virus antigens (106).

Therefore, we still do not understand whether the strong immune activation observed in DRESS is the cause or the consequence of herpes virus reactivation (69) and this may question the most adequate therapy. Most groups favor immediate drug suspension and corticosteroids to reduce the immune reactivation, whereas others prefer avoidance of immunosuppressors (43).

Effector Mechanisms in AGEP

Lymphocyte transformation tests, and, typically, patch tests are positive and, show a pustular pattern similar to the acute reaction with the typical spongiform intraepidermal pustule as in the acute eruption (34,52,54). Drug-specific T-cell clones isolated from these patch tests as well as from the blood are CD8+ and mainly CD4+ memory effector T cells, which exhibit cytotoxicity against drug-laden target cells, through perforin/granzyme B, granulysin, and Fas ligand (110). They secrete mainly a type 1 cytokine pattern [IFN-γ, TNF-α, and Ganulocyte–monocyte colony-stimulating factor (GM-CSF)], in some cases with IL-5, responsible for eosinophilia observed in about one third of AGEP patients (33,110). Nevertheless, the main particular characteristic of these T cells is the high production of CXCL8 (IL-8) and other cytokines, such as GM-CSF, that recruit CXCR1+ neutrophils to the epidermis where they will fill in the pustules. Moreover, other T-cell mediators, such as GM-CSF, INF-γ, and IL-17, acting mainly through the CXCR2, prevent neutrophil apoptosis and prolong their skin survival in the skin.

Preceding neutrophil skin infiltration, drug-specific CD4+ T cells (with less than 30% CD8+), expressing CCR6 as the skin homing receptor, are attracted to the skin and exert some cytotoxicity in the epidermis, important for the initial vesicle formation. The involvement of NK cells and granulysin is not very significant (42). As both T cells and keratinocytes secret CXCL8 and T cells also express the CXCR1, there is further T-cell activation by CXCL8 produced by keratinocytes, and therefore an amplification loop (52,110,69). Opposing MPE, there is a much lower expression of HLA-II by keratinocytes and no exotaxin was observed in epidermis, but only along endothelial cells (33).

This very peculiar pattern of drug-specific T-cell reaction, considered a type IVd hypersensitivity reaction (11), develops with drugs that usually induce other type IV reactions, namely, aminopenicillins. No reason has, thus far, been found to justify why some patients and in which circumstances a drug can elicit this particularly CXCL8-rich T-cell activity.

Effector Mechanisms in SJS/TEN

In exanthematic necrolysis, as Roujeau calls the group of diseases characterized by more than 1% epidermal necrolysis and detachment, including SJS, overlap and TEN, there is extensive epidermal apoptosis induced by T cells and their mediators (55).

Initially, T-cell participation was questioned as most biopsies do not contain lymphocytes in the dermis, but then T cells, mainly cytotoxic, were found in high numbers in the blister fluid of these patients (58), and earlier at the dermoepidermal junction and epidermis. These CD8+CD56+ CTLs are drug specific and kill autologous lymphocytes and keratinocytes in a drug and HLA restricted manner (59), and occasionally kill keratinocytes in the absence of drug (103). NK cells are also an important part of the cellular infiltrate in TEN (±13% of NKp46+ cells) and may also have an important part in keratinocyte necrolysis (apoptosis and some necrosis) (42).

These cells exert a direct cytotoxic effect by cell-to-cell contact or close proximity, with perforin/granzyme B and granulysin killing keratinocytes whose HLA-class I was modified by the drug. But the relatively small number of cytotoxic cells could hardly explain the exuberant epidermal necrolysis (55,102).

Therefore, apart from granzyme B and perforin from cellular granules, other soluble mediators found in blister fluid may amplify keratinocyte death. The suicidal interaction between Fas/Fas ligand (CD95/CD95L), in its membrane bound or soluble forms, was implicated as there is an increase in soluble Fas (sFas)

in blister fluid and blood of TEN patients (111) and FasL is upregulated in keratinocytes, but in skin biopsies Fas+ cells do not colocalize with FasL+ keratinocytes (112), i.v. immunoglobulins used in TEN based on their capacity to neutralize Fas had irregular results and the use of antibodies anti-Fas in animal models did not prevent keratinocyte apoptosis (13). Other molecules that may promote keratinocyte death are TNF-α and other death receptor ligands found in high amounts in blister fluid, particularly tumor necrosis factor (TNF)-related apoptosis-inducing ligand produced by CD8+ cells and TNF-like weak inducer of apoptosis produced by CD14+ and CD1a+ DC found in blister fluid (102).

At present the main candidate for disseminated epidermal necrolysis is granulysin, detected in blister fluids in very high amounts (>300-fold higher than in burn lesions), and with correlation with skin severity. These concentrations are capable of directly inducing keratinocyte apoptosis in vitro or in vivo. Moreover, only granulysin depletion in blister fluid abrogated its capacity to induce keratinocyte apoptosis (103). Also granulysin-producing cells (CD8+ and NK cells) accumulate in areas of more extensive epidermal aggression (42).

Apart from their initial epidermal aggression, CD8+ cytotoxic T secrete IFN-γ, which enhances disease spreading turning keratinocytes more susceptible to apoptosis and capable of inducing apoptosis of neighboring cells (59). Even without carrying the culprit drug, INF-γ-stimulated keratinocytes become more susceptible to killing by CD8+ T cells and TNF and its related death ligands, respectively, through upregulation of HLA class I molecules and death receptors (59,102). INF-γ-induced secretion of CCL27/CTACK further attracts CCR10+ cutaneous memory T cells (104) and cytokines liberated by damaged keratinocytes, namely, IL-18, also amplify the inflammatory loop (112), particularly in the absence of CD4+ CD25+Foxp3 regulatory T cells that do not migrate to the skin and are almost absent in regions of high CD8+ infiltration and marked apoptosis (108). In animal models of drug-induced TEN epidermal necrolysis is significantly reduced in the presence of Tregs, whereas CD4 T-cell depletion enhances necrolysis (113).

Although much is already known on pathomechanisms in SJS/TEN, the factors that drive the CADR into an SJS or TEN are not known, namely, the predilection for skin and mucosal aggression, eventually extending to epithelial cells in pharynx, bronchial, and gut tissue. TEN-inducing drugs are not different from those that induce other CADR, and at the beginning the skin reaction is similar to a MPE. Increased serum levels of sFas or granulysin may suspect the progression to a more severe life-threatening reaction, (70) and some authors suggest that, in individuals who develop SJS or TEN, lymphocytes have an increased capacity of secrete sFas, even in basal conditions (22).

Other genetic susceptibility markers, namely, HLA-B*1502 is highly associated with TEN from carbamazepine in some Asian populations, particularly if they also carry the specific Vβ-11 TCR, but other HLA molecules that confer susceptibility to severe drug eruptions do not discriminate between TEN, DRESS, or MPE, as HLA-B*5801 and allopurinol (personal data) (9).

Effector Mechanisms in Fixed Drug Eruption

FDE is a very peculiar drug hypersensitivity restricted to specific skin areas that reactivate on further drug exposure, due to reactivation of specific T cells that persist in residual lesions for many years (69).

Memory effector CD8+ T cells, with surface and activation markers of NK cells, namely, the CD69, persist in the epidermis in abnormal numbers for >4 years after clinical resolution of the acute lesion. Expression of the skin homing receptor (CLA+) and the integrin α3β7 (CD103), which binds E-cadherin in keratinocytes and IL-15 derived from lesional epidermis maintains their survival even in the absence of antigenic stimulus (60,114,115). In the resting plaque these intraepidermal T cells do not harm the neighboring keratinocytes, which are protected from apoptosis, but within a few hours upon exposure to the culprit drug these resting or "preactivated" T cells initiate a process of epidermal aggression. They upregulate mRNA for IFN-γ and secrete this cytokine in high amounts and initiate keratinocyte apoptosis, similarly to TEN, although more restricted: Fas-ligand binds Fas on keratinocytes and TNF-α, perforin, granzyme B, and granulysin secreted by these cells and other CD8+ effector T cells recruited from the circulation also participate in the epidermal aggression (114–116). Apart from CD8+, NK cells expressing high levels of granulysin accumulate at the dermal–epidermal interface where vacuolar degeneration is most significant and, as in TEN, these cells invade the epidermis and accumulate near areas of vesiculation or blisters although in a smaller amount (42).

As the reaction progresses CD4+ T cells, particularly CD4+CD25+^{hi} regulatory T cells, localize preferentially in the dermis and downregulate the reaction either by direct cell contact or by secretion of IL-10 or TGF-β (117). Contrary to FDE, in TEN the absence of these regulatory T cells can explain the extension of this apoptotic process, which has many similarities with the acute aggression observed in FDE (115). These regulatory T cells also seem to be involved in the process of desensitization in FDE (118).

The presence of the "preactivated" T cells in the residual lesional epidermis can explain why patch testing is negative in normal skin, whereas a few hours after application of the culprit drug in a residual lesion reactivation occurs with the clinical and histopathology typical of an FDE (62,115). Although some authors suggest these lesions can be reactivated by nonspecific stress/danger signals (60), in our experience lesional reactivation by patch testing is drug specific and allows the confirmation of the culprit drug and study cross-reactions (61,62,119).

IMMUNE-MEDIATED PATHOMECHANISMS AND IMPLICATIONS FOR CADR MANAGEMENT

The correct recognition of the main clinical patterns of immune-mediated CADR and the knowledge of the precise mechanisms involved is crucial for all the other procedures in the management of the patient.

In immediate reactions, with urticaria or angioedema suspected to be IgE-mediated care should be taken to avoid acute systemic symptoms, which might be life-threatening as oropharyngeal and laryngeal edema with respiratory distress or systemic symptoms of anaphylaxis that need prompt treatment with steroids and adrenaline while keeping vital signs. For the diagnosis of the culprit drug, provocation tests are not recommended in severe reactions and diagnosis should rely on in vitro tests for the detection of specific IgE (RAST/CAP) or basophil activation tests (BAT) with its different aspects (CD63 or CD203c expression) or skin prick or intradermal tests with immediate readings. Although with a relatively high specificity when correctly performed they carry a variable sensitivity (21). With drugs, such as penicillins and cefalosporins,

it is important to know which drug epitope is involved as this may determine the pattern of cross-reactive drugs, which is extremely useful for advising future antibiotic treatments. Eventually, hyposensitization may carefully be tried in trained centers.

In delayed exanthematous CADR in the acute phase it is important to look for clinical and laboratorial markers of poor prognosis. Bulla with Nikolsky's skin, reflecting CD8 and NK-mediated epidermal apoptosis, in a localized asymmetric and very well-limited area is observed in FDE, whereas more extensive involvement occurs in SJS/TEN, which is not always easy to distinguish from generalized bullous FDE; facial edema, high fever, and lymphadenopathy may suggest the multiorgan involvement in DRESS and pustules mainly in body folds may reflect the intense neutrophilic epidermal infiltration mediated by drug-specific T cells secreting high amounts of CXCL-8 in AGEP.

Simple laboratory tests to look for leukopenia or leukocytosis with atypical lymphocytes and eosinophilia (knowing this can occur later), thrombocytopenia as well as hepatic cytolysis or renal dysfunction have to be repeated along the course of TEN or DRESS to recognize severe complications.

In TEN, knowing the mechanisms of keratinocyte apoptosis, several studies are looking at serum concentrations of granulysin or Fas that seem to correlate with disease severity and, therefore, might be used as prognostic markers, influence therapeutic measures, and evaluate their efficacy. Although it is not exclusive for TEN, knowing that granulysin is one of the main mediators of keratinocyte apoptosis, some drug or monoclonal antibody that might abrogate its effects would be a promising therapy for this life-threatening CADR. Chung et al. showed that antibodies neutralizing granulysin contained in TEN blister fluid prevent keratinocyte apoptosis in vitro (103).

The search for viral reactivation in DRESS may give important clues for the definitive diagnosis of this CADR and, eventually, also on advantages of using specific antiviral therapies. Moreover, if drugs that cause viral reactivation in DRESS are precisely defined they should be completely avoided, at least until full recovery of this syndrome, which may take many months.

For a confirmation of the culprit drug in vitro tests that recognize specific T-cell activation (lymphocyte transformation test (LTT), lymphocyte activation tests or the IFN-γ ELISPOT assay) can be performed both during the acute phase and later for a retrospective diagnosis (69). Still, knowing the main cytokines produced by drug-specific T cells, both the evaluation of cytokine expression by flow cytometry or the ELISOPT can be improved by using these other markers of T-cell activation apart from IFN-γ, namely, IL-5, IL-13, granzyme B (101,21) and, eventually, granulysin. It is, nevertheless, important to remember that the T-cell response in in vitro tests may be hindered during the acute phase of DRESS due to an increase in Treg cells, as shown by the group of Shiohara, but that it becomes persistently positive after DRESS resolution (43).

Skin tests advised for delayed CADR are patch tests and, when negative, intradermal tests with delayed. Although they are highly specific when correctly performed, unhappily their sensitivity is rather low and depends on the pattern of drug eruptions and the culprit drug. They are frequently positive in DRESS and other MPE from carbamazepine, whereas they are always negative in allopurinol (32). Knowing that in residual lesions of FDE preactivated drug-specific CD8+CD56+ T cells persist for years, lesional patch testing with reading at 24 hours can be an important tool to confirm a diagnosis, therefore avoiding oral rechallenge that might reactivate all lesions and induce some new ones (62).

Patch testing has been a wonderful tool to study the pathomechanisms involved in CADR as drug-specific T cells isolated from them behave in a similar way as those collected from blood or skin during the acute episode. This may explain why often patch tests show clinical and histologic aspects very similar to the acute eruption as we have shown for TEN, AGEP, and DRESS (35).

Patch testing in abacavir hypersensitivity has been an important tool to confirm another important step in the knowledge of pathomechanisms in CADR related with pharmacogenetics (48). At present the very strong association with HLA-B*5701, and the cost-effectiveness of searching for this haplotype, has turned mandatory this exam before beginning therapy with abacavir. Similar steps are occurring with carbamazepine with pre-evaluation for HLA-B*1501 in Han Chinese to prevent SJS/TEN, a diagnosis that can be even more accurate with an additional search for the specific Vβ-11 TCR (55). At present we are also studying the cost-effectiveness of pre-evaluating HLA-B*5801 in our population before the administration of allopurinol, which in our experience is associated with frequent and severe CADR.

CONCLUDING REMARKS

Although much is already known on pathomechanisms in immune-mediated CADR that can be relevant for our daily practice, many steps are still not understood, namely, why, upon systemic i.v. or oral exposure, drug-specific T cells develop a particular skin migratory capacity, most of the time exclusively harming the skin. It is not known whether the drugs have particular skin affinity and combine with skin cells/proteins to be specifically recognized by circulating/resident skin cells that upregulate the inflammatory reaction, or if some unspecific activation of the innate skin immune system contributes to a further specific T-cell intervention against skin cells combined with the drug. Also it is difficult to explain why, as in viral exanthematous diseases, drug reactions usually develop from head to toes or affect only very limited areas of skin, as in FDE.

Still it is not known, apart from genetic repertoire of TCR or HLA molecules that predispose for certain drug eruptions, what triggers sensitization to the drug, which drug epitopes are recognized by the immune system so that cross-reactions are better understood and patients are better informed on drugs to avoid in the future. But even though there are no animal models to study the immune mechanisms, apart from a mouse model for TEN, news on this matter will certainly appear as many groups are actively working on this growing field on interest.

Moreover, we must recognize that the study of drug-induced hypersensitivity mechanisms can be important to recognize mechanisms in other diseases, as the discovery of CXCL8+ producing T-cell clones in AGEP stimulated the study of their contribution in other neutrophil-rich inflammatory skin diseases, such as psoriasis, Sweet's syndrome, Beçhet's disease, and have given immunologists the suggestion to consider a new type IV hypersensitivity reaction (IVd) (11).

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10 Systemic allergic (contact) dermatitis

Jeanette Kaae, Niels K. Veien, and Jacob P. Thyssen

INTRODUCTION

Systemic contact dermatitis (SCD) is a relatively uncommon and poorly understood aspect of allergic contact dermatitis (ACD) that highlights the potential for long-lasting immunologic memory in previously sensitized and nonsensitized areas of the skin. It may occur in persons with contact sensitivity after exposure to the hapten by oral exposure, transcutaneously, intravenously, or by inhalation. The condition can present itself with clinically characteristic features or be clinically indistinguishable from other types of contact dermatitis.

Contact sensitization to ubiquitous haptens is common. In a recent Danish population-based study, 10% reacted to one or more of the haptens in the standard patch-test series (1). The total number of individuals at risk of developing SCD is therefore large. SCD following exposure to medicaments is well established and there is increasing evidence for similar reactions from plant derivatives and metals, such as nickel, cobalt, and gold (2).

In our view, the term SCD may be somewhat misleading, as previous skin contact to the hapten in question is not always observed. We therefore believe that further confusion about the proper term should be avoided in the future and humbly suggest that the condition could be referred to as "systemic allergic dermatitis" (SAD). The background for this will be briefly discussed in the text. The use of the term is also an editorial decision of the journal "*Contact Dermatitis*".

The first description of SAD can probably be ascribed to the pioneering British dermatologist Thomas Bateman (3). His description of the mercury eczema called *eczema rubrum* is similar to what we today describe as the baboon syndrome.

Eczema rubrum is preceded by a sense of stiffness, burning, heat, and itching in the part where it commences, most frequently the upper and inner surface of the thighs and about the scrotum in men, but sometimes it appears first in the groins, axillae, or in the bends of the arms, on the wrists and hands, or on the neck.

The literature on SAD now includes reviews by Cronin (4); Fisher (5); Veien et al. (6); Menné et al. (7); Veien and Menné (8); and Thyssen and Maibach (9).

CLINICAL FEATURES

The clinical symptoms related to SAD are summarized in Table 10.1. The symptoms usually appear exclusively on the skin, although general symptoms are occasionally seen, for example, malaise and fever. Knowledge of the clinical symptoms stems from clinical observations and experimental oral challenge studies. Flare-up reactions of dermatitis in the primary site of contact dermatitis or previously positive patch-test sites should raise the suspicion of SAD (Fig. 10.1) (10,11,2,12).

Furthermore, flare-up of previously positive patch-test sites following ingestion of the hapten is a fascinating and specific sign of SAD. It is seen in relation to SAD from medicaments and in experimental oral provocation studies. Such flares of patch-test sites have not been a feature of the clinical spectrum of SAD. This symptom is hapten specific and can be seen years after patch testing. Christensen et al. (13) and Hindsén et al. (2) examined the specificity of this symptom in nickel-sensitive individuals. Positive patch tests to nickel and to the primary irritant sodium lauryl sulfate were made on previously unaffected skin areas. After several weeks, the individuals were given an oral nickel dose. A flare of dermatitis was seen at the nickel patch-test sites, but not at the sites of irritant dermatitis. Vesicular hand eczema (pompholyx or acute and recurrent vesicular hand eczema) (14), a pruritic eruption on the palms, volar aspects and sides of the fingers, and occasionally the plantar aspects of the feet, presents itself with deep-seated vesicles and sparse or no erythema. If the distal dorsal aspects of the fingers are involved, transversal ridging of the fingernails can be a consequence.

Erythema or a flare of dermatitis in the elbow or the knee flexures is a common symptom of SAD (15) and it is therefore difficult to distinguish from the early lesions of atopic dermatitis. Furthermore, flexual psoriasis can be a Köbner phenomenon associated with SAD.

The baboon syndrome (16) is a well-demarcated eruption on the buttocks, in the genital area and in a V-shape on the inner thighs with a color ranging from dark violet to pink. It may occupy the whole area or only part of it. Nakayama et al. (17) referred to the same syndrome as mercury exanthema. Lerch and Bircher (18) added acute, generalized exanthematous pustulosis to the syndrome. Based on case stories, the patients may have had SAD. Even extensive patch testing fails to confirm the diagnosis of SAD in some patients who show symptoms of the baboon syndrome as the same symptoms can have the same etiology. A nonspecific maculopapular rash is often part of a SAD. Even cases of vasculitis presenting itself as palpable purpura have been seen (19).

In relation to oral provocation with nickel or medicaments, general symptoms, such as headache and malaise, have occasionally been seen in sensitized individuals. In neomycin- (11) and chromate-sensitive patients (20), oral provocation with the hapten caused nausea, vomiting, and diarrhea and a few patients complained of arthralgia. However, the available information on the general symptoms observed in relation to the SAD reaction is anecdotal and deserves systematic documentation.

TABLE 10.1
Clinical Aspects of Systemic Allergic Dermatitis Reactions

Dermatitis in areas of previous exposure
Flare-up of previous dermatitis
Flare-up of previously positive patch-test sites
Skin symptoms in previously unaffected skin
Vesicular hand eczema
Flexural dermatitis
The baboon syndrome
Maculopapular rash
Vasculitis-like lesions
General symptoms
Headache
Malaise
Arthralgia
Diarrhea and vomiting

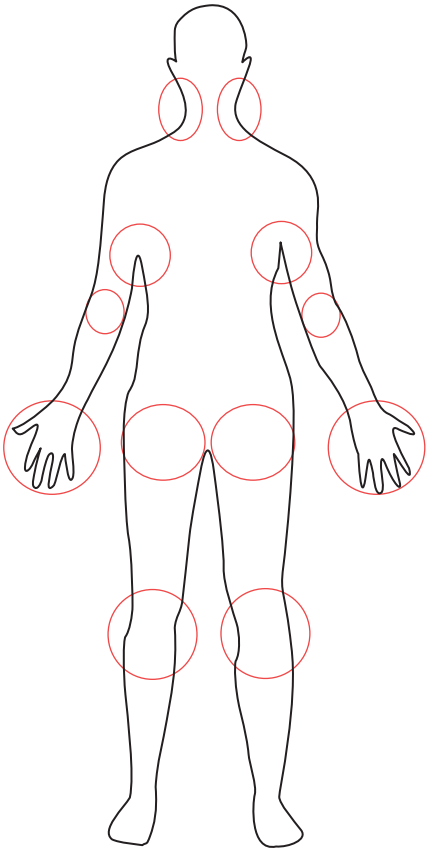

FIGURE 10.1 Summary of the typical localizations where eruptions are observed in patients with systemic allergic dermatitis.

DEFINITION OF SYSTEMIC DERMATITIS REACTIONS

Häuserman et al. presented terms to describe the development of contact allergy due to systemic administration of a hapten and challenged the baboon syndrome as a SAD reaction (21). They suggested that five new diagnostic criteria were required for a new syndrome called symmetrical drug-related intertriginous and flexural exanthema (Table 10.2), which was introduced to distinguish drug-elicited cases from other subtypes of systemic absorption of allergens, such as nickel and mercury (21).

IMMUNOLOGY/MECHANISM

Traditionally, the chain of events for SAD are as follows. A previous event of ACD due to a well-defined contact allergen occurs. The hapten, its metabolite, or an immunochemically related hapten is administered systemically and elicits a cutaneous reaction (22).

Based on human and animal experiments, it appears that both the humoral and the cellular immune systems are activated. The histopathology of the flare-up reaction is similar to that observed in ordinary ACD reactions (13).

Flares at sites of previous dermatitis or previously positive patch-test sites are probably caused by specific sensitized memory T cells, either resting at the site or homing to the area after specific hapten exposure. The investigation of lymphocyte subsets in the gastrointestinal mucosa and in blood before and after oral challenge with nickel in nickel-sensitized women showed a reduction of CD4+ cells, CD4+CD45Ro+ cells, and CD8+ cells in the peripheral blood of women with evidence of SAD. Oral challenge with nickel-induced maturation of naive T cells into memory cells that tended to accumulate in the intestinal mucosa (23).

Jensen et al. (24) found a reduction in the number of CLA+ CD45Ro+ CD3 and CLA+ CD45Ro+ CD8 but not CLA+ CD45Ro+ CD4 in the peripheral blood of nickel-sensitive patients after oral challenge with nickel. After the removal of a metal joint prosthesis containing cobalt, CD4 T-cell clones reacted to cobalt but not to nickel (25).

Möller et al. (26) challenged 10 patients allergic to gold with an intramuscular dose of gold and saw flare-ups of one-week-old gold patch-test reactions in all of them. Five also experienced a maculopapular rash, and four had transient fever. Plasma levels of TNF-α, IL-1ra, and sTNF-R1 and C-reactive protein were increased, particularly in those with fever.

In a later study of 20 gold- and 28 nickel-allergic patients challenged orally with nickel and gold in a double-blind, double-dummy fashion, three of 9 nickel-sensitive patients reacted to 2.5 mg nickel, whereas none reacted to gold. Six of 10 gold allergic patients reacted to 10 mg gold sodium thiomalate, whereas none of them reacted to nickel. TNF-R1 was increased in the plasma of nickel-sensitive patients challenged with nickel, whereas TNF-R1, TNF-α, and IL-1 were increased in gold-sensitive patients challenged with gold (27). In a study of 42 patients with SAD from *Toxicodendron*, it was suggested that a toxic rather than a specific immune reaction might be responsible (28).

The mechanism behind skin symptoms unrelated to previous contact dermatitis sites has been minimally evaluated. Veien et al. (29) investigated 14 patients with positive nickel patch tests and severe dermatitis. All were challenged orally with 2.5 mg nickel. After 6–12 hours, five developed widespread erythema. No clinical dermatitis developed in the erythematous areas. In a passive immunodiffusion assay, three of the five demonstrated precipitating antibodies in their sera against a nickel–albumin complex.

MEDICAMENTS

Pharmacologic Actions (The p-i Concept)

Most medications require covalent binding to proteins before becoming immunogenic. Additionally, many medications require metabolization before they can form a hapten–carrier complex; this process is often mediated by hepatic cytochrome P450 enzymes

TABLE 10.2
Criteria Proposed for Symmetrical Drug-related Intertriginous and Flexural Exanthema

Criteria	Description
1	Exposure to a systemically administered drug, first or repeated doses (contact allergens excluded)
2	Sharply demarcated erythema of the gluteal/perianal area and/or V-shaped erythema of the inguinal/perigenital area
3	Involvement of at least one other intertriginous fold
4	Symmetry of affected areas
5	Absence of systemic symptoms and signs

but may also take place in the skin (30). It has been suggested that the pathomechanism in SAD is similar to that in ACD (31). After metabolism of the causative drug in the skin, a hapten–carrier complex is processed by antigen-presenting cells and leads to clonal expansion of T cells in the local lymph node (31). T cells end up in the skin and develop a cytotoxic response (31). Posadas et al. suggested a different pathomechanism; that certain small-sized medications do not form hapten–carrier complexes but may instead bind directly to T-cell receptors without the presentation by major histocompatibility complex molecules. The binding is reminiscent of the pharmacologic interaction of drugs with immune receptors and is therefore called the "p-i concept" (32). This concept may explain why adverse cutaneous drug reactions and possible SAD are observed in some individuals at their first exposure to a medication (30). Based on these observations, we suggest that SCD is referred to as SAD in the future, as skin contact with the hapten is not a requirement for the development of SAD.

Antibiotics

Neomycin and bacitracin are widely used topical antibiotics. Contact allergy to these compounds is particularly frequent (4–8%) in patients with leg ulcers albeit the epidemiology is beginning to change. Ekelund and Möller (11) challenged 12 leg-ulcer patients sensitive to neomycin with an oral dose of the hapten and reported that 10 of 12 patients experienced a reaction. Five patients observed flares of their original dermatitis; six flares at the sites of previously positive patch tests; three developed vesicular hand eczema for the first time; and four patients experienced various gastrointestinal symptoms. Some surgeons use oral neomycin before colon surgery and even though neomycin is poorly absorbed from the gastrointestinal tract, severe SAD might occur in neomycin-sensitive individuals (12).

Contact sensitivity to penicillin was previously common, and flares of dermatitis have been seen in sensitized persons following exposure to traces of penicillin in milk (33). Contact sensitivity and SAD caused by penicillin can still occur after the topical use of the drug in the middle ear, in the peritoneum during abdominal surgery (16), or after occupational exposure. Tagami et al. described a patient with toxic epidermal necrolysis after the systemic administration of ampicillin and reviewed 10 other patch-test proven cases of dermatitis of similar morphology caused by various medications (34). Penicillin, ampicillin/amoxicillin, and erythromycin have been described as causes of SAD with baboon-like clinical features (35,36).

TABLE 10.3
Oral Challenge with Sulfonyl Urea Hypoglycemic Drugs in Sulfanilamide-Sensitive Patients

Substance	Allergen Dose (mg)	Duration of Treatment	Response
Carbutamide	500	Single exposure	7/25
Tolbutamide	500	Single exposure	3/11
Chlorpropamide	500	Single exposure	1/20

Antihistamines

The pharmacologic effectiveness of topically applied antihistamines is questionable. Antihistamines derived from ethanolamine and ethylenediamine are the most common contact-sensitizing antihistamines in the United States (37). Ethylenediamine-based antihistamines may elicit SAD in patients sensitized to ethylenediamine. Aminophylline, which contains theophylline and ethylenediamine, may elicit reactions in ethylenediamine-sensitized patients (38–40). Much of the knowledge in this field is based on anecdotal therapeutic accidents. In view of the relatively large number of persons who are contact sensitized to ethylenediamine, incidents of SAD to ethylenediamine derivatives must be considered rare.

Para-Amino Compounds

Sidi and Dobkevitch-Morrill (41) studied cross-reactions between *para*-amino compounds. Systemic reactions were seen after oral challenge with procaine in sulfonamide-sensitive patients, after challenge with *p*-aminophenylsulfamide in procaine-sensitive patients, and after challenge with *p*-aminophenylsulfamide and procaine in *p*-phenylenediamine-sensitive patients. Oral challenge with the sulfonyl urea hypoglycemic drugs in patients sensitized to *para*-amino compounds (sulfanilamide, *para*-phenylenediamine, and benzocaine) resulted in flare-up reactions in sulfanilamide-sensitive patients, but not in *para*-phenylenediamine and benzocaine-sensitive patients (Table 10.3). Oral challenge with tartrazine (20 mg) and saccharine (150 mg) in patients sensitized to *para*-amino compounds and sulfonamide did not produce any flare-up reactions (42,43).

Corticosteroids

Contact allergy to glucocorticoids is not uncommon in patients with eczematous skin diseases (44). The frequency seems to vary from center to center depending on local prescribing habits, degree of patient selections, and the diagnostic method used. Intradermal testing may offer additional diagnostic possibilities.

Patients sensitized to hydrocortisone may react with SAD when provoked orally with 100–200 mg hydrocortisone (45,46). Lauerma AI et al. and Torres V et al., these authors also investigated whether cortisol produced in the adrenals (i.e., hydrocortisone) could provoke SAD. In a placebo-controlled study, a patient was challenged with an adrenocorticotropic hormone stimulation test. A skin rash similar to that seen after oral hydrocortisone developed after 8 hours. Räsänen and Hasan (47) studied five patients who developed rashes when treated with

systemic or intralesional hydrocortisone. They recommend patch testing and intradermal testing to make the diagnosis of systemic hydrocortisone sensitivity and, if these tests fail, an oral challenge. Whitmore (48) reviewed 16 studies with a total of 24 patients who had SAD from corticosteroids. Typical clinical features were exanthema, localized dermatitis, generalized dermatitis, and purpura. Onset was often hours to days following ingestion of the corticosteroids. As a part of her thesis on corticosteroid allergy, Isaksson (49) challenged 15 budesonide-sensitive patients with 100 and 800 μg budesonide or placebo by inhalation. Four of seven challenged with budesonide had reactivation of previously positive patch-test sites as well as popular exanthema or a flare-up of previous dermatitis. Pirker et al. (50) saw an anaphylactoid reaction after inhalation of budesonide in a patient who was contact sensitized to budesonide. In another study, a betamethasone-sensitive patient developed baboon syndrome after the oral administration of betamethasone (51).

Miscellaneous Medications

Antabuse (tetraethylthiuram disulfide) is of particular interest, because it can cause SAD in three ways. Antabuse is used in the manufacture of rubber, as a fungicide, and in the treatment of chronic alcoholism. In patients sensitized to thiurams from the use of rubber gloves, systemic exposure to Antabuse can give rise to SAD (52). Subcutaneous implantation of Antabuse led to contact sensitization in two patients. Subsequent oral challenge with the hapten produced a flare-up reaction in one of the two patients. A similar patient was described by Kiec-Swierczynska et al. (53). Severe recall dermatitis of the penis was seen in a thiuram-sensitive patient after Antabuse treatment. He had been sensitized by the use of a rubber condom (54). Antabuse also induces SAD by an entirely different mechanism. As Antabuse and its metabolites are strong metal-chelating substances, they can cause systemic contact reactions in nickel- and cobalt-sensitive patients via a pharmacologic interaction in a dose-dependent manner (55–58).

Experimental oral challenge with 1 mg nickel before and during disulfiram treatment of a nickel-allergic patient showed greatly increased urinary nickel excretion during disulfiram treatment. A corresponding flare-up of dermatitis was seen (59).

The antitumor antibiotic mitomycin C can be used for the treatment of superficial bladder cancer. Colver et al. (60) demonstrated delayed-type hypersensitivity in 13 of 26 patients who had received mitomycin instillations by applying the allergen as a patch test (60).

de Groot and Conemans (61) reported six cases where intravesical administration of the drug resulted in SAD, including vesicular eczema of the hands and feet and dermatitis of the buttocks and genital area. A more widespread rash was eventually seen. Calkin and Maibach (62) reviewed delayed hypersensitivity to drugs and mentioned several patients who had positive patch tests to drugs and reactions to oral challenge with the same substances. Other medications associated with SAD are listed in Table 10.4.

TABLE 10.4
Medicaments that have Caused Systemic Allergic Dermatitis

Acetylsalicylic acid (77)
Aminophylline
5-Aminosalicylic acid (78)
Amlexanox (79)
Ampicillin
Antihistamines
Butylated hydroxy anisole, butylated hydroxy toluene
Cinchocaine (80)
Clobazam (81)
Codeine (82)
Corticosteroids
Diclofenac (83)
Dimethyl sulfoxide (84)
Ephedrine (85)
ε-Aminocaproic acid (86)
Erythromycin (87)
Estradiol (88)
Gentamycin
Hydromorphone (89)
Hydroxyquinoline (11)
Immunoglobulins (90)
8-Methoxypsoralen (91)
Mitomycin C
Neomycin
Norfloxacin (92)
Nystatin (93,94)
Panthothenic acid (95)
Penicillin (96)
Phenobarbitol (97)
Pristinamycine
Pseudoephedrine (98,99)
Pyrazinobutanzone (100)
Resorcinol (101)
Streptomycin
Sulfonamides
Tetraaethylthiuram disulfi de (Antabuse®)
Tramadol (102)
Vitamin B1 (103)
Vitamin C (104)

Note: Only references not mentioned in the text are listed in the table.

NICKEL

Contact sensitivity to nickel is common, particularly among young females (Fig. 10.2; (1)). Nickel-sensitive individuals seem to run an increased risk of developing hand eczema, particularly of the vesicular type (63). Christensen and Möller (10) showed that oral intake of nickel induces a SAD reaction in nickel-sensitive individuals. This observation led to intense research in the area of nickel dermatitis and SAD (64,65). Daily nickel intake varies from 100 to 800 μg (66,67). The highest nickel content is found in vegetables, nuts, whole wheat or rye bread, shellfish, and cocoa. Nickel exposure from drinking water, air pollution, and cigarettes is usually negligible, although exceptions occur (68). Certain makes of electric kettles and coffee machines and some glazed tea mugs may release significant amounts of nickel (69,70). Intravenous fluids may be contaminated with 100–200 μg Ni/L (71).

Only 1–10% of ingested nickel is absorbed. Nickel absorption varies greatly. Ingestion of 12 μg Ni/kg 1 hours prior to eating a 1400 kJ portion of scrambled eggs gave a 13-fold higher serum concentration of nickel compared with the simultaneous ingestion of nickel and scrambled eggs (72). Both fecal and urinary nickel excretion can be used as parameters of systemic nickel exposure. The nickel concentration in sweat can be high, ranging from 7 to 270 μg Ni/L (73,68,74). Christensen and Möller (10)

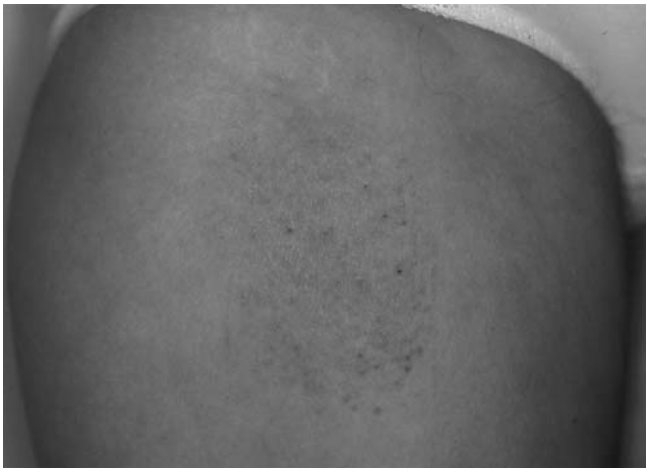

FIGURE 10.2 Site of previous allergic nickel dermatitis due to nickel release from metallic suspenders. Following oral nickel exposure, eruptions were noted on the thighs.

challenged 12 nickel-sensitive female patients with an oral dose of 5.6 mg nickel given as nickel sulfate. Nine of the patients developed flares of the dermatitis with crops of vesicles on the hands. The reaction appeared within 2–16 hours after ingestion. This observation has been confirmed (Table 10.5), and there is a marked dose–response relationship. Only a few nickel-sensitive patients react to oral doses of less than 1.25 mg of nickel, whereas most react to doses of 5.6 mg. A positive challenge test includes one or more of the previously described symptoms. The flares seen at former nickel patch-test sites are also dose-dependent (75) and are correlated to the intensity of the previous patch-test reaction and to the length of time since patch testing (2). There was rapid elimination of nickel in the urine after i.m. injection of nickel in hamsters, while elimination after cutaneous application of nickel was slow. Keratinocytes retained nickel much longer than did fibroblasts (76).

The clinical implication of these findings is uncertain (105,106). The nickel doses used in the challenge studies often exceed the amount of nickel in a normal daily diet. In experimental studies, flare-up reactions at sites of previously positive nickel patch tests have often been described. This phenomenon has not been observed in clinical practice. After oral challenge with 0.6–5.6 mg nickel typically given as nickel sulfate, a nonphysiologically high concentration of urinary nickel was observed on the days following the challenge (20–200 µg Ni/L). In two studies (107,108) involving a small number of patients, higher nickel excretion in the urine tended to be related to active hand dermatitis, but the urinary nickel levels were much lower than the concentrations measured on the days following oral nickel challenge. These observations do not exclude the possibility that systemic exposure to nickel is important for the chronicity of hand eczema related to nickel sensitivity. Undoubtedly, the daily nickel intake will sometimes exceed 0.6 mg, and two of five patients reacted to this dose in a study carried out by Cronin et al. (109). A rather unpleasant diet with a high nickel content has been shown to increase the activity of chronic nickel dermatitis (110). A diet with low nickel content may diminish the activity of hand eczema in some nickel-sensitive patients (111), and a flare of hand eczema has been seen in patients who abandoned such a diet (112).

CHROMIUM, COBALT, AND OTHER METALS

Sidi and Melki (122) suggested that oral dichromate ingestion in chromate-sensitive patients might be of importance for the chronicity of their dermatitis. This hypothesis has been tested in the studies listed in Table 10.6. Fregert (123) challenged five chromate-sensitive patients with 0.05 mg chromium given as potassium dichromate. Within 2 hours they developed severe vesiculation of the palms. One of the patients experienced acute exacerbation of generalized dermatitis. Schleiff (124) observed flares of chromate dermatitis in 20 patients challenged with 1–10 mg potassium dichromate contained in a homeopathic drug. Some of the patients also experienced flares in previously positive dichromate patch-test sites. Kaaber and Veien (20) studied the significance of the oral intake of dichromate by chromate-sensitive patients in a double-blind study. Thirty-one patients were challenged orally with 2.5 mg chromium given as potassium dichromate and a placebo tablet. Nine of the 11 patients with vesicular hand eczema reacted with a flare of dermatitis within one or two days but did not react to the placebo. Three patients experienced vomiting, abdominal pain, and transient diarrhea after the chromate challenge, but not after challenge with the placebo.

A SAD reaction to chromium has been seen after inhalation of welding fumes containing chromium (125), after the ingestion of a homeopathic drug (126), after a nutritional supplement with chromium picolate (127) and after ingestion of chromium in a multivitamin/multimineral tablet (128).

Compared with chromium and nickel, cobalt is well absorbed from the gastrointestinal tract. This makes cobalt-sensitive individuals candidates for further study of the possible existence of SAD caused by this metal (120). In a double-blind study, six of nine patients with positive patch tests to cobalt reacted to oral challenge with 1 mg cobalt given as 4.75 mg cobalt chloride (129). Most of the patients had recurrent vesicular hand dermatitis. Glendenning (130) observed a 49-year-old housewife with persistent eczema of the palms and isolated cobalt allergy. After the removal of metal dentures made of a cobalt–chromium alloy (Vitallium), dermatitis cleared. The patient had not had symptoms of stomatitis. Flare of cobalt dermatitis has been seen as a recall phenomenon in chronic alcoholics treated with tetraethylthiuram disulfide (131). Systemically aggravated contact dermatitis has been caused by aluminum in toothpaste in children who have been sensitized to aluminum in vaccines (132). There have been several reports of widespread exanthema or multiforme-like erythema in patients with positive patch tests to mercury compounds (133). Vena et al. (134) described 9 such patients, seven of whom also had systemic symptoms, such as malaise, pyrexia, and leukocytosis. The sensitization was induced by an antiparasitic powder that was thought to cause SAD after inhalation. Mercury in homeopathic medicine caused baboon syndrome in a 5-year-old girl (135).

Another route of systemic exposure is via dental treatment following the drilling of amalgam fillings. Following such treatment, a widespread maculopapular rash was seen in one mercury-sensitive patient (138), two patients developed nummular dermatitis (139), while another had flexural dermatitis (140) and one also had a flare of dermatitis at the site of a 4-week-old patch test to mercury (141). Flexural dermatitis is another manifestation of SAD in mercury-sensitive patients. A careful study of the concentration of mercury in saliva, feces, blood, plasma, and urine showed increased levels of mercury in saliva, blood, and feces during the first week after the removal of amalgam fillings. After removal of all the

TABLE 10.5
Challenge Studies in Nickel-Sensitive Patients with an Oral Dose of Nickel Given as the Sulfate

Author	Type of Study	Allergen Dose (Elementary Nickel) (mg)	Duration of Dosing	Response Frequency
Christensen and Möller (1975) (10)	Double blind	5.6	Single exposure	9/12
Kaaber et al. (1978) (113)	Double blind	2.5	Single exposure	17/28
Kaaber et al. (1979) (55)	Double blind	0.6	Single exposure	1/11
		1.2	Single exposure	1/11
		2.5	Single exposure	9/11
Veien and Kaaber et al. (1979) (114)	Open	4.0	Single exposure	4/7
Jordan and King (1979) (115)	Double blind	0.5	Two repeated days	1/10
Cronin et al. (1980) (109)	Open	0.6	Single exposure	1/5
		1.25	Single exposure	4/5
		2.50	Single exposure	5/5
Burrows et al. (1981) (116)	Double blind	2.0	Two repeated days	9/22
		4.0	Two repeated days	8/22
Percegueiro and Brandao (1982) (117)	Single blind	2.8	Repeated dose	34/43
		5.6		
Sertoli et al. (1985) (118)	Open	2.2	Single exposure	13/20
Gawkrodger et al. (1986) (119)	Double blind	0.4	Two repeated days	5/10
		2.5	Two repeated days	5/10
		5.6	Single dose	6/6
Veien et al. (1987a) (120)	Double blind	2.5	Single exposure	55/131
Santucci et al. (1988) (121)	Open	2.2	Single exposure	18/25
Hindsén et al. (2001) (2)	Double blind	1.0		2/10
		3.0		9/9

TABLE 10.6
Challenge Studies in Chromate-Sensitive Patients with an Oral Dose of Chromium Given as Potassium Dichromate

Author	Type of Study	Allergen Dose (Given as the Metal Chromium) (mg)	Duration of Dosing	Response Frequency
Fregert (1965) (123)	Open	0.05	Single exposure	5/5
Schleiff (1968) (124)	Open	1–10	Single exposure	20/20
Kaaber and Veien (1977) (20)	Double blind	2.5	Single exposure	11/31
Goitre et al. (1982) (135)	Open	7.1–14.2	Repeated exposure	1/1
Veien et al. (1994b) (136)	Double blind	2.5	Single exposure	17/30

Note: Eleven patients with pompholyx.

amalgam fillings, plasma mercury concentrations fell to 40% of the pretreatment level (142).

SAD from implanted metals is rare with the currently employed technology within orthopedic surgery. Case reports indicate that SAD may still occur in a sensitized patient after the insertion of a metal prosthesis. Guidelines for the diagnostic workup of such patients were recently published (143).

Orthodontic appliances have been seen to cause urticaria and dermatitis in nickel-sensitive persons (144–146). In some nickel-sensitive patients, the diagnosis required oral challenge with the metals nickel, cobalt, and chromium (147).

Möller et al. (148) challenged 20 gold-sensitive patients with sodium thiomalate or placebo. One of 10 who received the active compound experienced flare-up of a previous contact dermatitis site. All 10 patients experienced a flare-up of their previous gold patch-test sites, and several patients had toxicoderma-like symptoms. In a later study, Möller et al. (149) saw a flare-up of previously positive gold patch-test sites and transient fever in five of the five gold-sensitive patients.

OTHER CONTACT ALLERGENS

SAD was described in *Rhus*-sensitive patients who had eaten cashew nuts (150). A case of perianal dermatitis occurred after the ingestion of cashew nut butter (151). A baboon-like eruption occurred 36 hours after the ingestion of a pesto sauce containing cashew nuts (152).

SAD has been seen in patients sensitive to balsam of Peru, which contains naturally occurring flavors. Hjorth (153) observed SAD in balsam of Peru-sensitive patients who had eaten flavored ice cream and orange marmalade. Veien et al. (112) challenged 17 patients sensitive to balsam of Peru with an oral dose of 1 g of

balsam of Peru. Ten patients reacted to balsam of Peru and one to a placebo. Hausen (154) reviewed 102 patients sensitive to balsam of Peru. Ninety-three reacted to one or more of 19 constituents. Eight who had reactions to coniferyl benzoate and benzyl alcohol had SAD. Three of these patients had hand eczema and three had widespread dermatitis.

Based on questionnaires mailed to the patients 1–2 years after the initiation of diet treatment, Veien et al. (155) reviewed 46 balsam-sensitive patients who had been asked to reduce their dietary intake of balsams. Sixteen of 22 (73%) who had reacted to 1 g balsam of Peru in a placebo-controlled oral challenge had benefit from a low-balsam diet compared with 3 of 10 (30%) who had shown no reaction to the oral challenge. Nine of 14 (64%) who were placed on a low-balsam diet, but who were not challenged orally, benefited from a low-balsam diet. Salam and Fowler (156) studied 71 perfume and balsam-sensitive patients retrospectively. The dermatitis of 21 of 45 patients who followed a low-balsam diet improved or cleared. The most commonly implicated foods were tomato, citrus, and spices. Niinimäki (157) challenged 22 patients orally with balsam of Peru in a placebo-controlled study. Eight patients reacted to balsam of Peru but not to the placebo, while four reacted to both balsam of Peru and the placebo or only to the placebo. Aggravation of vesicular hand eczema was the most common clinical response. Similarly, Niinimäki (158) challenged 71 patients sensitive to balsam of Peru with spices. Seven had positive reactions to the challenge. Most had vesicular hand eczema.

Dooms-Goossens et al. (159) described SAD caused by the ingestion of spices in a patient with a positive patch test to nutmeg and in two patients sensitive to plants of the Compositae family after the ingestion of laurel. Sesquiterpene lactones found in Compositae caused SAD in a patient following the ingestion of lettuce (160). Goldenrod in an oral medication (Urodyn®) caused SAD in a 52-year-old man (161).

German chamomile tea caused a widespread eruption and anal pruritus in a 26-year-old woman who was sensitive to sesquiterpene lactone (162) and caused recurrent facial dermatitis in another patient (163). Inhalation of the allergen costus resinoid caused a baboon-like eruption in a sesquiterpene lactone-sensitive woman (164). A 45-year-old man developed widespread dermatitis after the ingestion of tea tree oil to which he had previously had a positive patch test (165). Kava extract caused SAD in one patient (166).

Garlic has been shown to cause SAD with vesicular hand eczema as the clinical manifestation. The dermatitis could be reproduced by placebo-controlled oral challenge (167). Ingestion of garlic has also caused SAD in the elbow flexures and periorbitally (168). Ophaswongse and Maibach reviewed cutaneous reactions following the ingestion of alcoholic beverages (1994). Both immediate- and delayed-type hypersensitivity reactions causing SAD were described. One patient became sensitized to ethanol in an estrogen transcutaneous delivery system. She developed widespread exanthema after the ingestion of alcoholic beverages (169).

The antioxidant butylated hydroxyanisole, which is used both in cosmetics and in foods, can cause SAD (170), as can substances as diverse as formaldehyde (171) and ethyl ethoxymethylene cyanoacetate (172). Preservatives such as sorbic acid have caused SAD appearing clinically as hand eczema (173,174).

Parabens have been suspected as the cause of SAD. However, only 2 of 14 paraben-sensitive patients experienced flares of their dermatitis after placebo-controlled oral challenge with 200 mg methyl and propyl parahydroxybenzoate. Both patients who reacted to the challenge had vesicular hand eczema (175).

RISK ASSESSMENT-ORIENTED STUDIES

Although the risk of SAD from drugs can be assessed, it is more difficult to carry out similar studies on ubiquitous contact allergens, such as metals and naturally occurring flavors. In spite of intensive research on the significance of orally ingested nickel in nickel-sensitive individuals, we are unable to give firm advice concerning the oral dose that would represent a hazard for the wide range of nickel-sensitive individuals. Many variables, such as the route of administration, bioavailability, individual sensitivity to nickel, interaction with naturally occurring amino acids, and interaction with drugs must be considered. A number of as yet unknown factors could influence nickel metabolism. Furthermore, immunologic reactivity to nickel can change with time and can be influenced by sex hormones and the development of tolerance. It is important to recognize that this area of research is extremely complex and that much well-controlled research is still needed. Jensen et al. (176) performed a modified meta-analysis of the theoretic risk of SAD after the oral administration of nickel in nickel-sensitive patients. The conclusion was that only a minority (<1%) of nickel-sensitive patients is at risk of SAD after the ingestion of nickel in food.

With regard to medicaments, it is possible to perform well-controlled oral challenge studies in sensitized individuals. The beta-adrenergic blocking agent alprenolol is a potent contact sensitizer. Ekenvall and Forsbeck (177) identified 14 workers employed in the pharmaceutic industry who were contact-sensitized to this compound. Oral challenge with a therapeutic dose (100 mg) led to a flare in one worker, who experienced pruritus and widespread dermatitis. Merthiolate is a preservative widely used in sera and vaccines. Förström et al. (178) investigated 45 merthiolate contact-sensitive persons to evaluate the risk of a single therapeutic dose of 0.5 mL of a 0.01% merthiolate solution given subcutaneously. Only one of the 45 patients developed SAD. Aberer (179) did not observe any reactions in a similar study involving 12 patients. Maibach (180) studied a group of patients who had discontinued the use of transdermal clonidine because of dermatitis. Of 52 patients with positive patch tests to clonidine, 29 were challenged orally with a therapeutic dose of the substance. Only one patient reacted with a flare-up at the site of the original dermatitis.

Propylene glycol is used as a vehicle in topical medications and cosmetics and as a food additive. Propylene glycol is both a sensitizer and a primary irritant. Hannuksela and Förström (181) challenged 10 contact-sensitized individuals with 2–15 mL propylene glycol. Eight reacted with exanthema 3–16 hours after the ingestion.

PATIENT MANAGEMENT

When medication-related SAD is suspected, the diagnosis relies on the patients' medical history, skin testing, and when indicated, oral challenge with the medication. Patch testing should not be performed within the first six weeks after the SAD (182) but not more than six months later. Patch testing has proved to be a valuable screening method, especially in patients with exanthema (183). Sometimes, depending on the use and need of the drug in question, negative patch test results should be supplemented by intradermal testing with delayed readings as a negative patch test

does not exclude the role of medications in causing the SAD reaction (101). However, when a positive patch test result is identified, it may be of past relevance and not related to the present adverse cutaneous drug reactions (101). The lymphocyte transformation test is an in vitro test that evaluates cell-mediated immunity through the proliferation of T cells after exposure to the chemical in question. It is considered both sensitive and specific and can sometimes be used as an adjunct to patch testing (184). An oral drug challenge can also be of diagnostic value in patients with negative skin tests, but it does not identify the involved pathomechanism and should only be performed if the drug is required for the patient's treatment of a disease. A recommended test method has previously been presented (185). It is generally considered safe to perform a drug challenge because a delayed-type immune reaction is suspected (8). Large patient materials from Finland have confirmed this and reproduced cutaneous symptoms in approximately 13% of the challenged patients (183).

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11 Immunologic mechanisms in allergic and irritant contact dermatitis

Iris S. Ale and Howard I. Maibach

INTRODUCTION

Contact dermatitis is an inflammatory skin condition induced by exposure to an environmental agent. It represents one of the most frequent types of human immunotoxicity. In the western world, 15–20% of the population has a contact allergy to one or more chemicals in their environment (1,2). It also results in significant morbidity with loss of time from work. In the USA, eczema and contact dermatitis (both irritant and allergic) account for 85–90% of all occupational skin disease (3). In addition, contact dermatitis frequently adopts a chronic and refractory clinical course, determining a substantial degree of physical handicap and compromise in the quality of life of the affected subjects.

Two main types of contact dermatitis may be distinguished: irritant contact dermatitis (ICD), due to direct proinflammatory effects of physical or chemical agents who are capable to activate the skin innate immunity; and allergic contact dermatitis (ACD), which is a T-cell-mediated hypersensitivity reaction, and therefore the inflammatory response is orchestrated by clonally expanded allergen-primed T lymphocytes. Thus, the main difference between both types of dermatitis is the presence of allergen-specific T cells as initiators of the inflammatory skin response in ACD. Other than that, the principal cellular and molecular pathways are essentially similar (4,5).

Recent advances in the pathophysiology of chemical-induced skin inflammation has shown that ICD and ACD are intimately associated and irritancy may predispose the development of contact sensitization. Hence, an efficient way to prevent ACD is to develop strategies to avoid ICD (6).

IMMUNOLOGIC MECHANISMS: ICD

As the outermost barrier of the human body, the skin is specialized to protect the individual from chemical, biological, and physical hazards from the environment. To induce a contact reaction, the harmful agent must penetrate the external aspect of the cutaneous barrier, that is, the stratum corneum (SC) and exert its effect on the viable skin layers. Most substances with irritant potential will damage the SC and expose the epidermal and dermal cells to their irritant effects, inducing the release of cytokines, chemokines, and other inflammatory mediators, which act as "danger signals" (7,8), stimulating the trafficking and infiltration of inflammatory cells and the activation of the innate immune system. Therefore, ICD represents an innate unspecific immunologic response triggered by the direct cytotoxic effect of the irritant. Barrier damage generates the initial alarm signal (9–12) and initiates a cytokine cascade that induces Langerhans cell migration and infiltration of monocytes, neutrophils, macrophages, and lymphocytes into the skin (13–17).

Interleukin (IL)-1α, IL-1β, and TNF-α are key cytokines in the inflammatory response induced by irritants (15–17). Preformed interleukin (IL)-1α, which is stored in the SC and the epidermis, probably starts the cytokine cascade. TNF-α increases major histocompatibility complex class II expression and intracellular adhesion molecule (ICAM) 1 expression on keratinocytes; playing a central role to uphold the inflammatory process (18). Some irritant reactions can be blocked by injection of anti-TNF-α antibodies or recombinant soluble receptors, (19). Therefore, polymorphisms in TNF-α genes may represent a risk factor for ICD (20–22).

Additional pro-inflammatory released molecules are IL-6, IL-8, CCL20, CCL27, IL12, and IL18, among others. So, the major pathophysiologic changes in ICD are skin barrier disruption, epidermal cellular changes, and cytokine release, all of which are interconnected.

IMMUNOLOGIC MECHANISMS: ACD

Chemically induced delayed contact hypersensitivity (CHS) is generally considered as a delayed-type hypersensitivity reaction primarily mediated by T cells. However, several characteristics of the reaction indicate that it is different from classic type IV delayed hypersensitivity. It is induced by chemical agents and metal ions, which penetrate the skin and form complexes with cutaneous proteins. This process is associated with a strong allergen-induced inflammatory reaction and leads to the migration of allergen-carrying dendritic cells (DC) from the skin to regional lymph nodes (LNs), where they induce the generation of allergen-specific T cells. The current paradigm of CHS follows a two-step mechanism, comprising an afferent or sensitization phase and an efferent or elicitation phase, which are considered to be temporally and spatially dissociated (23).

The clinically inapparent sensitization phase involves the events subsequent to the first contact with an allergen and it is complete when the individual is effectively sensitized. In this phase, epidermal Langerhans cells or dermal DCs become activated by the haptenated protein and migrate via the afferent lymph vessels to the skin-draining LNs, where they interact with and present the processed haptenated protein (the antigen) to naive T lymphocytes (24). This leads to the generation of skin-homing CD8+ Tc1/Tc17 and CD4+ Th1/Th17 effector T cells and memory T cells, which thereafter circulate in the blood and lymph vessels (25). Effector T-cell recruitment into the skin in order to elicit the response to

hapten challenge requires prior CXCL1-directed neutrophil infiltration and is dependent on interferon-γ (IFN-γ) and IL-17 produced by the hapten-primed T cells (26,27). The sensitization step lasts 8–15 days, and is thought to have no evident clinical consequence. In the elicitation phase, the re-exposure to the causative agent in sensitized individuals leads to the recruitment and activation of the effector T cells, which rapidly accumulate in allergen-exposed skin where they release proinflammatory cytokines and destroy allergen-loaded cells by cytotoxic activity, triggering the inflammatory process responsible for the cutaneous lesions (28). The interactions between T cells and antigen-presenting cells can take place directly in the epidermis, thus hastening the inflammatory process. The inflammatory reaction persists during several days and progressively decreases upon physiologic downregulating mechanisms. Although DC and effector T cells play a central role in the sensitization and elicitation phase of ACD, respectively, other cell types, including keratinocytes, NK cells, mast cells, and B cells contribute to the pathogenesis of the disease.

HAPTENS: CHEMICAL REACTIVITY AND PROINFLAMMATORY PROPERTIES

The stimulating agents in the CHS reaction are low-molecular weight chemicals called haptens, which are able to penetrate the skin. Most haptens are small compounds with a molecular weight of <500 Da. As haptens are too small to be immunogenic, they must be able to react with carrier macromolecules in the skin (usually considered to be proteins), to serve as antigens for the adaptive immune system (hapten-carrier concept). Thus, protein reactivity is mandatory for a chemical to become a contact allergen. Protein reactivity will allow not only for the formation of T-cell epitopes but, also, for the activation of the innate immune system.

Some nonreactive chemicals, so-called prohaptens, may access the xenobiotic metabolism pathway in the skin and turn into reactive contact allergens by enzymes of the cytochrome P450 family in skin cells, such as keratinocytes and dendritic DCs (29). Subsequently, the immunogenic chemicals may be exported out of the cell via multidrug resistance-related family proteins (30). Another mechanism for the generation of reactive contact allergens is the (auto-)oxidation of prehaptens (31).

IRRITANT PROPERTIES OF CONTACT ALLERGENS

A prominent attribute of contact allergens is their irritancy or adjuvanticity, that is, their ability to activate both innate immune and stress responses that seems to provide danger signals for efficient T-cell priming. Most known chemical allergens possess both sensitizing and irritant properties, triggering a local inflammatory response within the first hours after administration (32–34). Following contact with the skin, haptens cause activation of skin cells resulting in the rapid production of a whole array of inflammatory cytokines. In every step of CHS development, several cytokines are expressed and involved in the recruitment and activation of DC precursors, in the migration of skin DC to draining LNs and the development of the allergen-specific T-cell response. IL-1β and IL-1α are mainly produced by LC, but also by keratinocytes within minutes after hapten contact (35). During the initiation phase of the immune response, IL-1β mRNA can be detected in the dermis and the epidermis (35). IL-1β induces the TNF-α production of keratinocytes, which in turn serves as a signal for the LC emigration (15,36,37). LC and dermal DC carry the allergen to the LN, and the spectrum of DC-derived cytokines determines T-cell activation and polarization (38). So, in both ACD and ICD, IL-1α and IL-1β, as well as TNF-α are released as primary alarm signals, triggering the release of secondary alarm cytokines and chemokines, such as CCL20, CCL27, and CXCL8 among others (39). In a comparative study using a human skin equivalent, nickel sulfate, potassium dichromate, and sodium dodecyl sulfate induced a similar concentration-dependent increase in the secretion of IL-1α, TNF-α, and chemokines (CCL20, CCL27, and CXCL8) (39).

The ability of contact allergens to induce ACD correlates to a great extent with their inflammatory potential. This irritant property of contact allergens is thought not only to be substantial in the sensitization and elicitation phase of CHS, (34) but, also, to be responsible for the concentration dependence of the response (40,41). This feature differentiates contact allergens from conventional peptide antigens, which usually require additional innate signals or supplementation of exogenous adjuvants to generate immune responses. In addition, hypersensitivity reactions to protein antigens do not exhibit strict dose dependence once threshold concentrations have been surpassed. It has been proposed that mast cells may be the mediators of the adjuvant effects of haptens. Mast cell deficiency resulted in impaired emigration of skin DCs to the LN and contact hypersensitivity was dramatically reduced in mice in the absence of mast cells (42).

IRRITANCY AND ALLERGENICITY: THE INTERPLAY BETWEEN THE INNATE AND THE SPECIFIC IMMUNE SYSTEM

Contact allergens cause skin inflammation by the activation of the innate immune system. This is attained by inducing innate immune and stress responses employing pathways that are also used by pathogens. This immune response results in inflammation and is a prerequisite for the activation of the adaptive immune system with tissue-specific migration of effector and regulatory T cells (43,44). Activation of the innate defense mechanism induces the expression of endogenous effector molecules, proinflammatory cytokines, such as IL-12, IL-4, and chemokines. These molecules, in turn, may contribute to elicitation of the adaptive immune response, thus providing a link between the innate and specific immune mechanisms. The innate inflammatory immune response is a prerequisite for the activation and shaping of the adaptive immune response (45,46).

It has recently been demonstrated that toll-like receptors (TLR) and the nucleotide oligomerization domain (NOD)-like receptors stimulate and tune the quality of the adaptive immune response, playing a role in CHS (47–49). Thus, contact allergens can induce ligands for TLR2 and TLR4, (48–51) as well as activate the NLRP3 inflammasome and the production of reactive oxygen species (52). Stimulation of DCs by specific TLRs endows them with the capacity to cross-present exogenously acquired antigens and prime antigen-specific CD8+ T cells to differentiate into cytotoxic T cells (53,54). TLR triggering results in the production of proinflammatory cytokines, including IL-6 and IL-12, as well as production of pro-IL-1β and pro-IL-18. These are important mediators in CHS. Pro-IL-1β and pro-IL-18 are processed to their mature and secreted forms by the inflammasome, a cytosolic protein complex containing the NLR NLRP3 and the adaptor protein ASC (55,56). The inflammasome is activated by contact allergens and then activates

caspase-1, which processes pro-IL-1β and pro-IL-18 (56–58). The absence of cytokines, such as IL-1β, IL-18, IL-6 or IFN-γ impairs or abrogates the induction of CHS in mice (57). On the other hand, the influence of anti-inflammatory cytokines, such as IL-10, produced by regulatory T cells, B cells, and mast cells also causes the inhibition of CHS development (59). Analysis of the cytokine spectrum in the different phases of CHS may allow the identification of a contact allergen-specific cytokine signature or signatures that will be useful in the identification of potential contact allergens.

CONCLUSION

The current understanding of the immunologic mechanisms of both ICD and ACD does not allow for establishing pertinent and practical criteria for a clear-cut differentiation between them. The crucial discernible difference is the involvement of allergen-specific T cells as initiators of the inflammatory reaction in ACD, but, the principal inflammatory pathways are essentially similar in both types of dermatitis. For a chemical to cause irritation or skin sensitization it must be able to gain access to the viable epidermis, and to produce local immunologic danger signals, activating the unspecific innate immune response.

The elucidation of the innate inflammatory pathways, cellular components, and mediators will help to identify new drug targets for more efficient treatment of contact dermatitis and hopefully also for its prevention. Further understanding of the molecular pathways in contact dermatitis would be significant in dermatologic practice as well as in toxicologic research.

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12 Allergic contact dermatitis: Elicitation thresholds of potent allergens in humans

Ludivine J. Bernard, J. J. Hostýnek, and Howard I. Maibach

INTRODUCTION

Contact dermatitis is an inflammatory condition caused by direct skin exposure to an offending chemical with or without a requirement for ultraviolet light. There are two distinct types of contact dermatitis: irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD). ACD, an eczematous disease mediated through immune mechanisms, is an acquired skin disorder that occurs at sites of contact with small chemical haptens in only those individuals who have been previously exposed to, and immunologically sensitized to a particular chemical. In contrast to ICD typically only a small percentage of the population develops an eruption when exposed to chemicals causing ACD. The most common chemical allergens causing the condition in North America include nickel sulfate ($NiSO_4$), as well as the pentadecylcatechols, the active moiety in plants of the genus *Rhus*, which include poison ivy, poison oak, and poison sumac (1).

An extensive literature discusses weak *versus* strong allergens. The term refers either to the percent of population sensitized or to molecular potency—the concentration of a chemical eliciting ACD. For the purposes of this chapter, strong allergens elicit a reaction at low concentration; weak allergens require a higher dose. This study creates a database of xenobiotics that have been described as human allergens of extreme molecular potency, eliciting reactions at low challenge concentrations. The resulting biologically based algorithm derived from analysis of the difference in structural alerts statistically associated with protein reactivity between the two categories (evaluating quantitative structure–activity relationship), may permit a priori classification of untested chemicals as strong or weak allergens.

The database of chemical structures known to cause ACD in humans that are classified by potency is limited compared with more than a thousand compounds considered as allergens in animals (2). Only a few dozens of allergens with extreme potency are known to cause ACD in humans. This might be because most dermatologists routinely test patients using the standard patch test series only at the single concentration recommended, which leads to an undifferentiated "yes"/"no" diagnosis for hypersensitivity. It may also be, however, that allergens that meet our criterion of "extreme" are uncommon. It is intuitive but not documented that with equal skin exposure, a potent (in ppm) allergen might be expected to sensitize more individuals than a less potent allergen.

MATERIALS AND METHODS

As the terms utilized here (potency, ppm, and so on) are not typically indexed, standard dermatologic journals were searched manually as well as through MEDLINE, from 1956 to the present. Manually searched journals included the following: *Contact Dermatitis*, *American Journal of Contact Dermatitis*, *Dermatitis*, *British Journal of Dermatology*, *Journal of American Academy of Dermatology*, *Archives of Dermatology*, *Acta Dermato-Venereologica*, *Acta Dermato-Venereologica (Supplements)*, and *Journal of Investigative Dermatology*.

Only human data were utilized. Standard patch testing, provocative use test, and repetitive open application test were used in the studies reviewed here. Keywords included human, ACD, elicitation, patch test, threshold concentration, metals, and biocides. References from retrieved papers were examined. Chemicals were included based on the following criteria:

1. Arbitrary maximum elicitation concentrations of 500 ppm (0.05%)
2. Defined chemical structure
3. A minimum record of two patients or volunteers to any given compound.

We do not differentiate between ACD (allergic dermatitis from relevant exposure) and contact allergy (a positive patch test of uncertain clinical relevance) because the literature does not generally permit such distinction.

RESULTS

Metals

Mercury

Mercury was recognized as a contact sensitizer in 1895. Exposure can occur with three chemical forms: metallic mercury, such as yellow oxide of mercury or mercury from a broken thermometer, mercury salts (phenylmercuric salts), such as, in tattoos containing the red pigment cinnabar (mercuric sulfate), and organic mercury, including thimerosal, methylmercury, and merbromin. The most common contact with mercury is through thimerosal, which is used as an antiseptic and preservative. Thimerosal may be found in topical medications, especially ophthalmic and nasal preparations, cosmetics, and as a preservative in vaccines. Methylmercury exposure occurs through consuming contaminated fish.

The *American Journal of Contact Dermatitis* identified thimerosal as the contact allergen (nonallergen) of the year in 2002. Although it is the fifth most frequently found contact allergen in patch-tested patients in the United States, the 1998–2000 North American Contact Dermatitis Group (NACDG) database reported thimerosal to have a definite or probable relevance in only 2.9% of the patients with a positive test. Because of this low clinical relevance frequency, the NACDG elected to delete thimerosal from its allergen testing "standard baseline tray" of patch-test allergens; however, it is still part of the T.R.U.E. TEST patch-test series.

Although avoidance of thimerosal-containing vaccines in patients with contact hypersensitivity may be considered, adverse effects other than a localized injection site reaction are rare. In patients with symptomatic oral disease, adjacent to mercury amalgams, patch tests with a dental series containing metallic mercury allergens may be appropriate (3).

In the human repeat-insult patch test, mercuric chloride ($HgCl_2$) caused 92% positive reactions, making the ionized form a class 5 (extreme) sensitizer on the Magnusson–Kligman scale (4). Metallic mercury (e.g., in amalgam), is only a moderate topical sensitizer.

Tested with $HgCl_2$, 0.05% (500 ppm; exposure time of two days, reading on day 3), 58 of 377 patients reacted; the number of positive reactions was significantly greater among patients with pierced ear lobes (29/107) than among those without piercing (27/270) (5). Patients with baboon syndrome (systemic eczematous contact-type dermatitis) and gold dermatitis due to ear lobe piercing were tested with 0.05% (500 ppm) $HgCl_2$ (patch tests applied for two days, reading on day 3); five of five patients with baboon syndrome were patch-test positive. Twenty-one of 35 patients who reacted positively to patch testing with mercury had pierced ears (6).

In 13 mercury-allergic patients, the mean threshold concentration of mercury has been evaluated in two different vehicles: distilled water and petrolatum.

- $HgCl_2$ 0.05% (500 ppm) in distilled water: patch tests were positive in two patients and negative in petrolatum.
- $HgCl_2$ 0.025% (250 ppm) in distilled water: patch tests were positive in three patients and in four patients in petrolatum.
- $HgCl_2$ 0.0031% (31 ppm) in petrolatum: patch tests were positive in two patients and negative in distilled water.
- $HgCl_2$ 0.0015% (15 ppm) in distilled water: patch tests were positive in three patients and in two patients in petrolatum (7).

The permissible time-weighted average (TWA) level of mercury vapor in the workplace is 0.05 mg/m^3 (8). Investigation of mercury sensitivity among health professionals revealed a sensitization rate of 2.4–7.2% (9).

In two cases of occupational exposure to mercury vapors at a level of 9.9 mg/m^3, medical professionals presenting with clinical symptoms of systemic sensitization reacted to challenge testing with 0.05% (500 ppm) aqueous (aq.) $HgCl_2$, one of them to 0.05% (500 ppm) thimerosal in petrolatum (10). When 12 patients with oral mucosal lesions associated with amalgam restorations were patch tested with aq. $HgCl_2$ 0.05% (500 ppm), five gave a positive skin reaction (patch tests applied for two days, read on day 3) (11).

Patients with oral mucosal lichenoid lesions due to amalgam restorative materials were tested with several organic mercury compounds. Reactions were read at 24 and 48 hours, and also after 3, 10, and 17 days. Of 19 patients, tests with 0.05% (500 ppm) phenylmercury acetate gave six positive reactions after three days, one on day 10 and one on day 17. The test with 0.05% (500 ppm) phenyl mercury nitrate was positive in two patients on day 3 and one on day 10. With 0.05% (500 ppm) thimerosal, one patient each was positive on days 3, 10, and 17 (12). Of 1025 contact dermatitis patients tested, 215 (21%) were positive to thimerosal overall, 12 (8%) to 0.05% (500 ppm) thimerosal in petrolatum, and 138 patients showed positive reactions to 0.05% (500 ppm) ethylmercury chloride in petrolatum (13). There is a suggestion of the high frequency of sensitization to thimerosal in atopic children. Of four children tested with serial dilutions of thimerosal in petrolatum (exposure time of two days, reading time on days 2 and 3), all showed a positive reaction at 0.1% (1000 ppm) and three also at 0.01% (100 ppm) (14).

The relative risk of sensitization to different classes of allergens was evaluated in a multicenter study involving 31,849 random eczema patients from 24 dermatology departments, including health care workers, tested over four years (1992–1995). Significantly higher sensitization rates overall were recorded among health care workers when compared with the control group of patients (not involved in health care-related occupations). Incidence of positives to phenyl mercuric acetate (0.01%) patch tests (exposure time 24–48 hours, reading time at 72 hours) was 4% of 1349 in the medical occupations *versus* 3.7% of 10,486 controls (15). Among 10,974 patients tested with phenyl mercuric acetate aq. 101 (1.7%) reacted to a concentration of 100 ppm (16).

A similar multicenter study evaluated sensitization rates to different series of preservatives, antimicrobials, and industrial biocides. Among 19,454 dermatology patients tested with preservatives of the standard series from 1990 to 1994, 4.0% reacted to thimerosal at 0.05% (500 ppm). Tested with a different preservatives series, 440 of 9361 reacted to 0.05% (500 ppm) thimerosal and 101 of 1852 to phenyl mercuric acetate (17).

In 24 dermatologic departments, 6548 randomly selected patients were tested over four years (1990–1994) for allergy to sodium timerfonate. At 0.05% (500 ppm) (exposure 24–48 hours, reading at 72 hours), 31 had positive reactions (17). In a series of studies, different cohorts of subjects who had previously given positive patch-test reactions to thimerosal were tested with ethylmercury chloride in ethanol and aq. methylmercury chloride. Test reading was done on days 2 and 4. A 0.0165% (165 ppm) ethylmercury chloride in ethanol resulted in 32 of 32 positives in a first study (17). In the second study, 36 of 36 patients had positive reactions to 0.031% (310 ppm) aq. methylmercury chloride, 18/18 to 0.031% (310 ppm) ethylmercury chloride in ethanol, and 18/18 to 0.015% (150 ppm) ethylmercury chloride in ethanol (18). In a third study, 19 of 21 patients had positive reactions to 0.0165% (165 ppm) ethylmercury chloride in ethanol and to 0.031% (310 ppm) aq. methylmercury chloride (19).

Gold

Gold can be described as a covert allergen. Since patch testing for gold hypersensitivity has recently become routine in North

America, Europe, and Japan, a considerable prevalence of patch-test positivity to gold has been noted. Even so, the degree of clinical relevance of such allergy remains questionable (20), since the condition usually remains "silent" or subclinical (21). This may explain why few attempts were made to evaluate skin reactivity to low gold concentration in patch tests. Gold sodium thiosulfate (GST) at 0.5% (5000 ppm) in petrolatum is the accepted standard for routine testing.

Defining the clinical relevance of positive gold patch tests is illustrated by the outcome of a patch-test study conducted among an important cohort of clinic patients and volunteers. Patches were removed after two days and read after 2 and 4 days. Of 1203, 38 patients were found positive to 0.05% (500 ppm) GST (3.2%), *versus* five of the 105 volunteers (4.8%), most of whom had no identified previous exposure to gold (22). In a separate study, these authors found eight patients out of 373 with a positive patch test to 0.05% (500 ppm) GST (2.1%) (23).

In a maximization test involving human subjects, hypersensitivity was induced by epicutaneous application of 2% gold chloride. On challenge with 0.005% (50 ppm) of the reagent, 16 of 23 gave a positive reaction (4).

Nickel

Nickel (Ni), recognized as a premier cause of ACD, belongs to the metals group that reacts with eccrine sweat and can form divalent Ni ions; these, in turn, can penetrate the stratum corneum via the transappendageal or transcellular route to reach the viable epidermis. Reacting there with aminoacid residues, the resulting Ni-complexed protein may then cause contact allergy (24).

The potential threshold for inducing Ni sensitivity due to contact with irritated skin, also a putative cause of so-called housewife hand dermatitis, was investigated by a hand-immersion experiment: upon exposure twice daily for 23 days to a surfactant solution, 12 of 20 individuals tested showed positive reactions to 10 ppm aq. $NiSO_4$, six of 12 to 5 ppm, three of 20 to 1 ppm, and two of 20 to 0.5 ppm. Also, there was a pronounced difference in reactivities depending on the test site (25).

An elicitation concentration of more than 100 ppm Ni (as nickel chloride) was necessary to elicit an allergic reaction in a cohort of Ni-sensitized individuals (26).

Three hundred thirty-two patients with previously diagnosed contact allergy to Ni or a history suggestive of Ni allergy were tested with serial dilutions of $NiSO_4$ (exposure 24–48 hours, reading at 72 hours or later) (27).

- $NiSO_4$ 0.0005% (5 ppm): patch tests were negative in all;
- $NiSO_4$ 0.001% (10 ppm): patch tests were positive in four of 92;
- $NiSO_4$ 0.005% (50 ppm): patch tests were positive in five of 92;
- $NiSO_4$ 0.01% (100 ppm): patch tests were positive in 19 of 329;
- The effect of repeated exposure of the hands to low Ni concentrations over two weeks was evaluated by patch testing postexposure in a study of 17 $NiSO_4$-sensitive volunteers, to simulate occupational exposure (28);
- Nickel chloride 0.02% (200 ppm): patch tests were positive in four patients;
- Nickel chloride 0.01% (100 ppm): patch tests were positive in four patients.

The suggestion was made that a more differentiated approach using dilution series is advisable for diagnostic purposes, rather than the current practice of applying the single 5% $NiSO_4$ patch. The reaction threshold to $NiSO_4$ was tested in 53 patients sensitized to Ni by patch testing with serial dilutions, both aqueous and in petrolatum. Reactivity was compared between cohorts sensitive to Ni only, and an equal cohort sensitized to both Ni and cobalt (Co) (29).

Threshold concentration $NiSO_4$ 0.039% (390 ppm) aq.: patch tests were positive in two patients sensitive to Ni only, one patient was sensitive to Ni and Co.

- $NiSO_4$ 0.039% (390 ppm) petrolatum: patch tests were positive in three patients sensitive to Ni only, five patients sensitive to Ni and Co.
- $NiSO_4$ < 0.039% (< 390 ppm) aq.: patch tests were positive in five patients sensitive to Ni only, four patients sensitive to Ni and Co.
- $NiSO_4$ < 0.039% (< 390 ppm) petrolatum: patch tests were positive in one patient sensitive to Ni only, two patients sensitive to Ni and Co.

We conclude the mean reaction threshold for $NiSO_4$ in water was lower (0.43%) than in petrolatum (0.51%). The lowest thresholds were observed in patients simultaneously sensitive to both Ni and Co.

Twenty-five Ni-sensitive patients were patch tested by the application of a dilution series of $NiSO_4$ (30). A 112 ppm Ni (0.05% $NiSO_4$) caused reactions in nine patients, 1.12 ppm in one of the patients tested.

The threshold of sensitivity in individuals with positive reactions to Ni was determined in a serial dilution test with $NiSO_4$ in petrolatum: of 35 tested, four individuals reacted to 390 ppm, 6–190 ppm, and 1–100 ppm (31).

Repeated patch testing with $NiSO_4$ at 0.0032% (32 ppm) was performed on the upper part of the back of 15 females. Tests were applied for two days and read on day 3 after application. Four reacted positively (32).

In 2008, patch testing was realized on the upper back of 20 people with serial dilution of $NiSO_4$ in petrolatum during 48 hours. The reading was on 96 hours after the application of the patch.

- At the dilution of 0.5 ppm $NiSO_4$, zero positive response was noted.
- At the dilution of 1 ppm $NiSO_4$, one positive response was observed.
- At the dilution of 5 ppm $NiSO_4$, two positive responses were elicited.
- At the dilution of 10 ppm $NiSO_4$, seven positive responses were elicited.
- At the dilution of 100 ppm $NiSO_4$, 10 positive responses were elicited. (33)

In another study, which took place between January and May 2008, 16 people were tested on the back with serial dilution of $NiSO_4$ in water. They performed patch test and strip patch testing:

- One positive response was induced with a strip patch test of 50 ppm $NiSO_4$.
- Eight positive responses were induced with a strip patch test of 100 ppm $NiSO_4$.

They conclude that strip patch test could help to detect hidden allergens if there is no positive reaction with patch testing (34).

Cobalt

Although cobalt is an essential trace element, its salts can pose significant dermatologic problems, primarily in the work environment, due to their allergenic potential. Hypersensitivity to cobalt is mostly associated with Ni hypersensitivity, because in metallurgy the two metals are often used in tandem; also due to their chemical similarity and problems in obtaining test materials free of Ni, patch testing for cobalt sensitization often leads to false-positive diagnoses.

Patch testing of 60 cobalt-allergic patients (including cobalt-sensitive only, cobalt- and Ni-sensitive, cobalt- and chromium-sensitive) gave the following results:

- Cobalt chloride 0.0312% (312 ppm) patch tests elicited eight positive reactions in distilled water and eight positives in petrolatum.
- Cobalt chloride 0.0156% (156 ppm) patch tests induced one positive reaction in distilled water and three positives in petrolatum.
- Cobalt chloride <0.0156% (<156 ppm) patch tests induced 14 positive reactions in distilled water and 14 positives in petrolatum (7).

Chromium

In the trivalent state (Cr^{3+}), chromium is an essential trace element, important for several biological processes in humans. Poorly soluble, Cr^{3+} salts are also poor penetrants through biological membranes, including undamaged human skin. As a Cr^{3+} compound, chromium trichloride hexahydrate can be used. In the hexavalent state (Cr^{6+}), mostly of anthropogenic origin, chromium is highly immunogenic, genotoxic, and carcinogenic in mammals. Its salts easily penetrate the skin, particularly in the work environment, thereby causing irritation and allergy of the delayed type. Potassium dichromate ($K_2Cr_2O_7$) can be used as a Cr^{6+} compound.

In a study of 47 chromium-allergic patients (including chromium-sensitive only, chromium- and cobalt-sensitive, chromium-, cobalt-, and Ni-sensitive), the mean threshold concentration of chromium has been evaluated in three vehicles: distilled water, petrolatum, and alkaline aq. buffer (pH 12).

- Potassium chromate 0.0312% (312 ppm) in distilled water: patch tests were positive in one patient, in buffer in eight patients, and in petrolatum in seven;
- Potassium chromate 0.0156% (156 ppm) in distilled water: patch tests were positive in seven patients, in buffer in five patients, and in petrolatum in three;
- Potassium chromate 0.0078% (78 ppm) in distilled water: patch tests were positive in two patients, in buffer in nine patients, and in petrolatum in two;
- Potassium chromate 0.0039% (39 ppm): patch tests were all negative, except for in one patient in buffer;
- Potassium chromate <0.0039% (<39 ppm) in distilled water: patch tests were positive in two patients, in buffer in eight patients, and in petrolatum in two (7) (Table 12.1).

In 2001, a study with 17 chromium-allergic volunteers was performed. They patch tested with 100, 10, and 1 ppm Cr and repeated open application test (ROAT) in two phases: they applied, twice daily, aqueous solution of potassium dichromate containing 1.0% of sodium lauryl sulfate (SLS) on the antecubital fossa, for a week. In the first phase, they applied a concentration of 5 or 10 ppm Cr and if there wasn't any response, they did the second phase with 20 and 50 ppm Cr.

- Patch test on normal back skin with 100 ppm Cr: patch test was positive on two patients at day 2 reading;
- Patch test on normal back with 10 ppm Cr : two were positive for patch test at day 2 reading and one at day 4;
- Patch test on normal back skin with 1 ppm Cr: any positive patch test;
- Patch test on SLS pretreated back skin with 100 ppm Cr: five positive for patch test at day2 reading, and six at day 4;
- Patch test on SLS pretreated back skin with 10 ppm Cr : one positive for patch test at day 2 reading, and three at day 4;
- Patch test on SLS pretreated back skin with 1 ppm Cr : two positive for patch test at day 4 reading;
- ROAT with chromium: three subjects out of 15 reacted to the concentration of 5 and 10 ppm;
- ROAT with chromium: two subjects out of 11 reacted to the concentration of 20 ppm Cr and three reacted to the concentration of 50 ppm Cr.

This study showed that Cr gives more allergic reactions than irritant reactions and repeated exposure to a low concentration of Cr can lead to an allergic reaction (35).

A 2003 study on chromium allergic patients demonstrated the following:

- minimum threshold elicitation 10% for Cr^{3+} was 6 ppm
- minimum threshold elicitation 50% for Cr^{3+} was 89 ppm
- minimum threshold elicitation 10% for Cr^{6+} was 1 ppm
- minimum threshold elicitation 50% for Cr^{6+} was 5 ppm

Thus, Cr^{3+}, which was not previously considered to play a significant role in chromium allergy should be re-evaluated. Both Cr^{3+} and Cr^{6+} are capable of eliciting dermatitis at low concentrations in the same patient (36).

Between January and May 2008, patch testing and strip patch testing were performed with serial dilutions with distilled water of potassium dichromate on the back of 17 volunteers.

- Three positive responses were elicited with a strip patch test of 50 ppm potassium dichromate;
- Seven positive responses were elicited with a strip patch test of 100 ppm potassium dichromate;
- Ten positive responses were elicited with a strip patch test of 500 ppm potassium dichromate;
- Four positive responses were elicited with patch test of 500 ppm potassium dichromate and all the four had reaction with strip patch test at a lower concentration.

They concluded that strip patch test could help detect hidden allergens if there is no positive reaction with patch testing (34).

TABLE 12.1
Positive Reactions to Metals

Materials	Screening Concentration	Results (Positive/ Tested Subjects)	References	Materials	Screening Concentration	Results (Positive/ Tested Subjects)	References
Mercuric chloride	500 ppm	29/207	(5)	Nickel sulfate	390 ppm	4/35	(31)
	500 ppm	27/270	(5)		<390 ppm aq.	5/53	(29)
	500 ppm	5/5	(6)		190 ppm	6/35	(31)
	500 ppm	21/35	(6)		112 ppm	9/25	(25)
	500 ppm	5/12	(11)		100 ppm	19/329	(27)
	500 ppm	2/2	(10)		50 ppm	5/92	(27)
	500 ppm aq.	2/13	(7)		32 ppm	4/15	(32)
	250 ppm aq.	3/13	(7)		10 ppm	4/92	(27)
	250 ppm pet.	4/13	(7)		10 ppm	12/20	(25)
	31 ppm pet.	2/13	(7)		5 ppm	6/12	(25)
	15 ppm aq.	3/13	(7)		1 ppm	3/20	(25)
	15 ppm pet.	2/13	(7)		0,5 ppm	2/20	(25)
Phenyl mercuric acetate	500 ppm	101/1852	(16)		100 ppm	10/20	(33)
	500 ppm	5.5%	(12)		10 ppm	7/20	(33)
	100 ppm	8/19	(16)		5 ppm	2/20	(33)
	100 ppm	192/10974	(15)		1 ppm	1/20	(33)
	100 ppm	1.7%	(15)	Strip patch test	100 ppm	8/16	(34)
		54/1349 (4%)			50 ppm	1/16	(34)
		388/10486 (3.7%)		Cobalt chloride	312 ppm aq.	8/60	(7)
					312 ppm pet.	8/60	(7)
					156 ppm pet.	3/60	(7)
Thimerosal	500 ppm	778/19454 (4.0%)	(16)		<156 ppm aq.	14/60	(7)
	500 ppm	440/9361 (4.7%)	(16)	Potassium chromate	312 ppm buff.	8/47	(7)
	500 ppm	12/1025	(13)		312 ppm pet.	7/47	(7)
	100 ppm	3/4	(14)		156 ppm aq.	7/47	(7)
					156 ppm buff.	5/47	(7)
					156 ppm pet.	3/47	(7)
Sodium timerfonate	500 ppm	31/6548 (0.5%)	(16)		78 ppm aq.	2/47	(7)
					78 ppm buff.	9/47	(7)
Ethylmercury chloride	500 ppm	138/1025	(13)		78 ppm pet.	2/47	(7)
	310 ppm	18/18	(17)		<39 ppm aq.	2/47	(7)
	165 ppm	19/21	(19)		<39 ppm buff.	8/47	(7)
	165 pm	32/32	(18)		<39 ppm pet.	2/47	(7)
	150 ppm	18/18	(17)		100 ppm	2/17	(35)
Methylmercury chloride	310 ppm	19/21	(19)		10 ppm	2/17	(35)
	310 ppm	36/36	(17)		100 ppm (SLS pretreated)	10/17	(35)
Phenylmercury nitrate	500 ppm	3/19	(12)		10 ppm (SLS pre treated)	4/17	(35)
					1 ppm (SLS pre treated)	2/17	(35)
Gold sodium thiosulfate	500 ppm	8/373 (2.1%)	(23)		5 ppm (ROAT)	5/15	(35)
	500 ppm	38/1203 (3,2%)	(22)		10 ppm (ROAT)	5/15	(35)
	500 ppm	5/105			20 ppm (ROAT)	2/11	(35)
	50 ppm	16/23	(4)		50 ppm (ROAT)	3/11	(35)
				Strip patch test	500 ppm	10/17	(34)
Nickel chloride	200 ppm	4/17	(28)		100 ppm	7/17	(34)
	100 ppm	4/17	(28)		50 ppm	3/17	(34)

Abbreviations: ROAT, repeated open application test; SLS, sodium lauryl sulfate.
Source: Adapted from Ref. 95.

Botanicals

Primin has been included in the European standard series of patch tests since 1985, as contact dermatitis from the plant *Primula obconica* became common in Western Europe and Scandinavia. Increasing the test concentration beyond 0.01% (100 ppm) was found unadvisable as reactions to primin 0.01% (100 ppm) are

already strong, and raising the concentration further would increase the risk of active sensitization by patch testing. Primin 0.01% (100 ppm) patch tests were positive in 57 of 3075 random dermatologic patients (37).

At the Department of Dermatology, Copenhagen University Hospital, Gentofte, 13,986 patients with suspected contact allergy were patch tested with the European standard series, from 1985 to 2004.

Patch test to primin 0.01% (100 ppm) in petrolatum were positive in 151 patients of 13 986 patients. Of 149 of these patients, 12 were men and 137 were women. However, the justification for inclusion in the standard patch series is questioned because of the diminution since 2000 of case of contact dermatitis due to *P. obconica* (due to the diminution of the production of this variety) (38).

Contact dermatitis due to plants of the Araliaceae family has been reported, and the major allergen in these plants was shown to be falcarinol. At 0.03% (300 ppm) falcarinol in petrolatum, patch tests elicited 4/4 positives in patients allergic to the Araliaceae family (39).

During a 16-year-period (May 1993 to May 2009), patch test at 0.03% (300 ppm) falcarinol in petrolatum was tested on the back of 127 patients for two days and was reading on days 3 or 4 and 7. Ten were positive (7.9%) and 23 were doubtful positive. Falcarinol is stable when it is in the refrigerator and in petrolatum. So it should be considered for plant test series worldwide (40).

Cross-allergy among these plant constituents has been determined in a study of sensitized Japanese farmers and of previously healthy control subjects who had been sensitized by patch testing. Compounds from the evergreen plants *Dendropanax trifidus* and *Fatsia japonica*, belonging to the family Araliaceae, led to the following patch tests results (read at days 2, 3, and 7) (41).

- 0.01% (100 ppm) *cis*-9,17-Octadecadiene-12,14-diyne-1,16-diol—5/5 positive
- 0.01% (100 ppm) 16-Hydroxy-*cis*-9,17-octadecadiene-12,14-diynoic acid—4/5 positive
- 0.01 % (100 ppm) *cis*-9, *trans*-16-Octadecadiene-12, 14-diynoic acid—2/5 positive

A 0.05% (500 ppm) *cis*-9,17-Octadecadiene-12,14-diyne-1,16-diol elicited six of seven positive reactions in patients allergic to the plants of Araliaceae family, and also in four of five control subjects (42).

Tulipalin A (α-methylene-γ-butyrolactone) was identified as the allergen in tulips and alstroemeria. Patch testing at 0.03% (300 ppm) in ethanol elicited three positive reactions out of three horticulturists presenting with skin lesions (43).

Another well-known plant allergen from urushi plants is urushiol. Patch testing with urushiol at 0.01% (100 ppm) in petrolatum (exposure time two days, reading on day 3) gave positive reactions in six of eight forest workers also allergic to 2,2′-azobis (2-aminopropane) dihydrochloride (44). There is a suggestion about cross-reactivity to urushiol in patients with contact dermatitis due to *Dendropanax trifidus* (Araliaceae family): four of seven patients showed strong positive reactions when patch tested with 0.01% (100 ppm) urushiol (42).

Dehydrocostus lactone, a component of Compositae mix, is often responsible for Compositae dermatitis in gardeners. Dehydrocostus lactone at 0.033% (330 ppm) petrolatum patch tests were positive in four of 10 Danish florists with occupational dermatitis (Table 12.2) (45).

TABLE 12.2
Positive Reactions to Botanicals

Materials	Screening Concentration (ppm)	Results (Positive/ Tested Subjects)	References
Primin	100	57/3075	(37)
Falcarinol	300	4/4	(39)
	100	151/13986	(38)
	300	10/127	(40)
cis-9,17-Octadeca-diene-12,	500	6/7	(42)
14-Diyne-1,16-diol	500	4/5	(42)
	100	5/5	(41)
16-Hydroxy-*cis*-9,17-	100	4/5	(41)
Octadecadiene-12,14-diynoic acid	100	2/5	(41)
Tulipalin A	300	3/3	(43)
Urushiol	100	6/8	(44)
	100	4/7	(42)
Dehydrocostus lactone	330	4/10	(45)
Chloroatranol	200 to 0.0063	13/13	(46)
	5 ppm (ROAT)	12/13	(46)
	200 ppm	10/10	(47)
Atranol	163 ppm	8/10	(47)

Abbreviation: ROAT, repeated open application test.
Source: Adapted from Ref. 95.

Chloroatranol (3-chloro-2.6-dihydroxy-4-methyl-benzaldehyde) was identified as a contact allergen in the natural fragrance extract, oak moss absolute, which is derived from the lichen *Evernia prunastri* and widely used in perfumery.

In 2003, a study was performed with 13 eczema patients known to be sensitized to both oak moss and chloroatranol and 10 control subjects who are eczema patients without sensitization to either material. Patch testing with serial dilutions of chloroatranol in ethanol from 220 to 0.0063 ppm, 10 dilution steps and ethanol, as vehicle control, were applied to the upper back during 48 hours and the reading was on removal of the patches, on days 2, 3, and 7. In each patient, one or more reactions were found for all dilutions. They performed use tests on the 13 patients: repeated open applications were made on the lower arm. They applied twice daily a solution of chloroatranol 5 ppm for two weeks and in case of no reaction, they continued another two weeks with a 25 ppm solution. They obtained positive use test in all the patients. Twelve subjects of the 13 became positive to the lowest concentration and one needed high concentration to react.

This study showed that the majority of the patients react with a minimal exposure. Chloroatranol is more potent than 5-chloro-2-methyl-4-isothiazolinone (MCI) and 2-methyl-4-isothiazolinone (MI). Chloroatranol is not the only allergen in oak moss absolute, atranol is also an allergen present in oak moss absolute. Atranol (2.6-dihydroxy-4-methyl-benzaldehyde) is present in a higher quantity than chloroatranol (46).

In 2006, a study was performed with choroatranol and atranol. They path tested with serial dilutions of chloroatranol (200 to 0.00063 ppm in ethanol) and of atranol (163 to 0.00052 ppm in ethanol, in equimolar concentration). Patch tests were applied on the upper back of the 10 eczema patients for two days and the readings were obtained on days 3 and 7. All the patients were diagnosed with contact allergy to chloroatranol within the past five years.

- Chloroatranol at 200 ppm (top concentration): 10 of 10 patients had a positive reaction.
- Atranol at 163 ppm (highest concentration): eight of 10 patients had a positive reaction.

For both the substances, there is a highly statistically significant dose dependence. Chloroatranol is a stronger allergen than atranol but there is more atranol in oak moss than chloroatranol and also in the perfume. The estimated concentration eliciting a reaction in half of the tested individuals (ED 50%) was 0.28 ppm for chloroatranol and 1.1 ppm for atranol. In view of these results, Scientific Committee on Consumer Products, an independent advisory committee to the European Commission recently recommended that chloroatranol and atranol should not be present in any cosmetic products (47).

Biocides

As a group, chemicals that have biocidal (antimicrobial) properties and are used as disinfectants, preservatives, pesticides, and are otherwise useful in deterring, reducing, or eliminating undesirable or dangerous organisms, have one characteristic in common: they are highly protein-reactive, and their activity is based on changing or destroying basic functions of target organisms. Since the demise of the effective and popular antibacterial hexachlorophene in the early 1970s, mainly the isothiazolinones MCI, MI, 1,2-benzisothiazolinone (BIT), and methyltrimethylene isothiazolinone (MTI), have taken its place. Applications for isothiazolinones as stabilizers of aqueous media have proliferated: preservatives in household and industrial products such as metal-working fluids, cooling-tower water, latex emulsions, paper mills, and most significantly for consumers, in cosmetics and toiletries. Commensurate with their rate of application, the literature reports that their allergenic action has also increased.

From 1983 to 1986, 365 patients with suspected sensitization to MCI/MI were patch tested with the biocide. Twenty patients had positive reactions at 100 ppm MCI/MI aq. and in petrolatum (48).

MCI/MI: 0.005% (50 ppm) patch tests were positive in 10 of 24 patients; at 0.0025% (25 ppm) patch tests were positive in 9 of 24 (49).

MCI/MI: 0.01% (100 ppm) patch tests elicited 24 of 24 positive reactions in patients believed to be allergic to the antimicrobial (49).

MCI/MI at 0.0015% (15 ppm) in the provocative use tests (PUT) in rinse-off products (six applications daily for 14 days): positive in three of 27 tested.

MCI/MI at 0.0025% (25 ppm) in the PUT in rinse-off products: positive in four of four tested.

MCI/MI 0.0015% (15 ppm) PUT in leave-on products (two applications daily for seven days): positive in 31 of 107 tested.

MCI/MI at 0.0007% (7 ppm) in the PUT in leave-on products (two applications daily for 14 days): positive in 52 of 52 tested.

MCI/MI at 0.015% (150 ppm) in the PUT in leave-on products (two applications daily for 14 days): positive in four of 567 persons tested (50).

Patch testing of seven individuals hypersensitive to MCI/MI with serial dilutions of the biocide gave the following results:

- 200 ppm of MCI/MI—seven of seven had positive reactions
- 100 ppm—five of seven reacted positively
- 50 ppm—four of seven, and 25 ppm—two of seven had positive reactions (51)

MCI/MI at 0.01% (100 ppm) patch tests, read on days 2, 3, and 4–7: positive in 15/590 patients with ACD; 0.02% (200 ppm) patch tests—positive in 16/589 patients (50).

MCI/MI as 0.01% (100 ppm) patch tests were positive in 3% of 3078 patients suspected with ACD (52).

MCI/MI as 0.01% (100 ppm) patch tests were positive in 2.5% of 2110 women with medical occupations (15).

Sixteen of 225 MCI/MI-sensitized subjects reacted to 100 ppm (53).

MCI 0.002% (200 ppm) patch tests gave positive reactions in two of 45 sensitized subjects. MTI at 0.03% (300 ppm), patch tests were positive in three of 19 (54). In random dermatologic population tested to 0.03% (300 ppm) octylisothiazolinone, seven of 1556 patients had positive reactions (55).

Of 1094, 4.2% children tested positive to patch testing with 100 ppm of MCI (56).

BIT as 0.04% (400 ppm) aq. patch, exposure time—2 days, reading on days 3 and 4: positive in four of 17 occupational contact dermatitis patients; 10 of 537 patch tests were positive (57).

BIT as 30 ppm patch: 10 of 556 patch tests were positive in random dermatologic population (58).

BIT as 0.05% (500 ppm) alcohol solution has been tested among employees at a manufacture of air fresheners (exposure time two days, reading on day 3): three of four workers showed positive reactions. In the same group, three of five workers reacted positively to 0.03% (300 ppm) Proxel CRL [ethylenediamine (24%) and BIT (23%) solution] (59).

In another study published in 2006, 29 eczema patients who had reactions to the standard patch test concentration (100 ppm aq.) of MCI/MI were recruited. They performed two ROATs of four weeks separated by four weeks washout period. During ROAT 1, they applied 2 ppm (0.025 μg/cm^2) of MCI/MI on the skin and during ROAT 2, they applied 7.5 ppm (0.094 μg/cm^2) of MCI/MI. Seven persons on 25 had a positive reaction during the ROAT 1 and 14 on the 25 had a positive reaction during the ROAT 2. They could conclude that allergic contact dermatitis with MCI/MI depends on time and dose per unit area (60).

Over a three-year period, in the U.K., 1524 patients were patch tested, 46 were positive at day 2 reading at 200 ppm MCI/MI. Thirty-five patients of these 46 were patch tested with MCI/MI 100 ppm. At day 4 reading, 19 patients had a positive reaction.

They demonstrated that 200 ppm did not cause any irritant reactions or cases of active sensitization. Sweden uses this concentration in their baseline series (61).

In 2005, MI alone was permitted in cosmetic products at a maximum concentration of 100 ppm.

In Finland, they documented the frequency of positive patch test reactions to MI, and its relevance to MCI/MI sensitivity during 2006 to 2008.

- Year 2006, 17 of 3981 (0.4%) had a positive reaction to 300 ppm of MI and 51 on 3851 (1.3%) a positive reaction to 100 ppm MCI/MI
- Year 2007, 20 of 3382 (0.6%) had a positive reaction to 300 ppm of MI and 71 of 3382 (2.1%) positive reaction to 100 ppm MCI/MI
- Year 2008, 32 of 3458 (0.9%) had a positive reaction to 300 ppm MI and 72 of 3458 (2.1%) person positive reaction to 100 ppm MCI/MI
- Thus, from 2006 to 2008, 10,821 were tested and 69 (0.6%) had a positive reaction to 300 ppm MI and 194 (1.8%) positive reaction to 100 ppm MCI/MI
- 33 patients did a use test for a maximum of two weeks: 10 were positive for MI 100 ppm and all of this 10 were positive to MCI/MI 100 ppm.

They concluded that MI alone can also elicit contact allergy (62).

Methyldibromo glutaronitrile (MDBGN) was introduced in the 1980s, as a preservative in cosmetics, household products and industrial products, often used in combination with phenoxyethanol (PE). In 2003, the European Commission banned the use of MDBGN in leave-on products. They limited its use to the rinse off products. One study performed a ROAT with three concentrations of MDBGN/PE: 50 ppm MDBGN (which is the lowest active biocidal concentration according to one manufacturer, 100 ppm, and 250 ppm. The ROAT was realized on the forearm of 39 volunteers. The dilution was applied twice daily, and leave on, during two weeks if there was no response, they applied the next higher concentration for two weeks.

- Thirteen patients on 39 reacted at 50 ppm MDBGN;
- Eight reacted at 100 ppm MDBGN;
- Three reacted at 250 ppm MDBGN.

Overall, 24 patients had a positive reaction. They documented that, in leave-on products, there was no concentration of MDGBN which can be safe for humans and have at the same time, microbicidal activity. In rinse off products, a study showed that, with regard to the elicitation, the safe concentration of MDBGN may be close to 200 ppm. (63).

A 2010 study on rinse off products utilized a ROAT in MDBGN-allergic patients with a liquid soap containing 50, 200, or 400 ppm of MDBGN. They used the first concentration twice daily for four weeks, if there was no reaction, they used the next higher concentration for four weeks. Five patients on 37 reacted positively: 1/37 react to the lower concentration 50 ppm, three reacted to 200 ppm and one reacted to a concentration of 400 ppm of MDBGN. They concluded that a concentration in rinse off products near 50 ppm may be safe for most individuals already sensitized and this concentration might prevent elicitation (64).

In a trial, the eliciting threshold concentration of formaldehyde in formaldehyde-sensitive individuals was studied by the occluded and nonoccluded patch test in serial dilution; also the relationship to ROAT with a product containing a formaldehyde releaser was evaluated. Formaldehyde at 0.025% (250 ppm) occluded patch test (exposure two days, reading at days 2, 3, 6–9) gave positive readings in three of four. No positive reactions were observed in the nonoccluded patch test or the ROAT (Table 12.3) (65).

TABLE 12.3
Positive Reactions to Biocides

Materials	Screening Concentration (ppm)	Results (Positive/ Tested Subjects)	References
MCI/MI	200	16/589	(50)
	200	40731	(51)
	150	4/567	(51)
	100	24/24	(50)
	100	15/590	(49)
	100	20/365	(50)
	100	92/3078 (3%)	(48)
	100	53/2110 (2.5%)	(52)
	100	40729	(53)
	100	16/225	(51)
	100	46/1094 (4.2%)	(57)
	50	10/24	(51)
	50	4/7	(54)
	25	9/24	(49)
	25	4/4	(51)
	25	2/7	(51)
	15	3/27	(49)
	15	33/107 (31%)	(50)
	7	52/52	(50)
	2	7/25	(60)
	7.5	14/25	(60)
	200	56/1524	(61)
	100	19/35	(61)
	100	51/3851	(62)
	100	71/3382	(62)
	100	72/3458	(62)
	100	194/10821	(62)
	100	10/33	(62)
MI	300	17/3981	(62)
	300	20/3382	(62)
	300	32/3458	(62)
	300	69/10821	(62)
	300	10/33	(62)
MDGBN	250	3/18	(63)
	100	8/26	(63)
	50	13/39	(63)
	400	1/37	(64)
	200	3/37	(64)
	50	1/37	(64)
Chloromethylisothiazolinone	200	2/45	(55)
Methyltrimethylene isothiazolinone	300	3/19	(55)
Octylisothiazolinone	300	7/1556 (0.4%)	(56)
1,2-Benziosothiazolin-3-one	500	3/4	(65)
	400	4/17	(58)
	400	10/537	(58)
	30	10/556	(59)
Proxel CRL [ethylenediamine (24%) and 1,2-benzisothiazolin-3-one (23%) solution]	300	3/5	(65)
Formaldehyde	250	3/4	(66)

Abbreviations: MCI/MI, 5-chloro-2-methyl-4-isothiazolinone/2-methyl-4-isothiazolinone.
Source: Adapted from Ref. 95.

TABLE 12.4
Positive Reactions to Corticosteroids

Materials	Screening Concentration (ppm)	Results (Positive/ Tested Subjects)	References
Budesonide	200	7/10	(67)
	100	20/26	(68)
	20	8/10	(68)
	10	19/26	(68)
	2	8/10	(68)

Source: Adapted from Ref. 95.

Corticosteroids

The allergenic response to low concentration of corticosteroids may be muted due to their anti-inflammatory properties, which can protect skin from inflammatory signs. Low concentrations of corticosteroids may nevertheless produce strong positive reactions (66) because an anti-inflammatory level may not have been reached. Budesonide 0.0002% (2 ppm) patch tests (occlusion time, 48 hours; reading time, days 2 and 4): 8/10 (80%) positive; 0.002% (20 ppm) patch tests—8/10 (80%) positive; 0.02% (200 ppm) patch tests—7/10 (70%) positive (59). For comparison, budesonide 2% (20,000 ppm) patch test with the same occlusion and reading time only elicited five positive reactions in 10 subjects tested (50%). In a different study, where patch tests also remained under occlusion for 48 hours and reading has been done on day 3, the results were as follows: budesonide 0.001% (10 ppm)—19 of 26 tested subjects reacted positive (73%); budesonide 0.01% (100 ppm)—20 of 26 subjects had positive reactions (78%) (Table 12.4) (67).

DISCUSSION

The database of chemical structures known to cause ACD in humans, which are classified by potency, is limited when compared with the more than thousand compounds thought to be allergens in animals (68–70). Of those, allergens known to be of extreme potency number only a few dozen. This may be because dermatologists routinely test patients using standard patch test series at a single concentration, which leads to an undifferentiated "yes"/"no" diagnosis for hypersensitivity. Only exceptionally do research dermatologists resort to statistically significant cohorts of human volunteers to establish threshold elicitation concentrations by dose–response design, or by multiple-dose response studies (25,29,71,72). It may also be, however, that allergens that meet our criterion of "extreme" are uncommon.

Furthermore, clear and unambiguous classification as to allergenic potency of a chemical per se is problematic, because it depends on several endogenous and exogenous factors:

- Clinical experience suggests the existence of different degrees of sensitivity among patients allergic to the same chemical. Several authors proposed a quantitative concept of "strong" *versus* "normal" or "weak" contact allergy (73). According to this concept the skin reactions to different concentrations of a chemical correlate with the grade of the previous sensitization, rendering an individual more (or less) likely to respond to skin contact with a given quantity of an allergen (74,75).
- Intraindividual variation in test reactivity (30).
- Presence (or absence) of active atopic dermatitis in a given individual (76).
- Degree of sweating (77–79).
- Age and gender (80–84).
- In addition to gender variability, there is the assumption that the patient's immunologic status might be influenced by and vary according to the stage in the menstrual cycle. Studies and case reports have shown increased test reactivity both to allergic and irritant reactions premenstrual (32).
- Anatomic site tested (32,85–87).
- Vehicle (88–90).
- Different patch test brands (91).
- Varying amounts of an allergen placed on the skin (92).
- Differences in testing; patch test, PUT, and ROAT (93).

We note other limitations of data correlation: additional variables include study population (routine screening of dermatitis patients *versus* aimed testing), literature collection (hand screening), and under-reporting.

Finally, there is a suggestion that the threshold for elicitation is not a constant property of an allergen. Remarkably, this effect is seen across species in guinea pigs, mice, and humans in a number of allergens (94). This is why it can be difficult to determine clear threshold doses that will or will not elicit allergic responses.

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13 Photoirritation (phototoxicity, phototoxic dermatitis)

Panthea Heydari, Natalie M. Moulton-Levy, and Howard I. Maibach

INTRODUCTION

Drug-induced skin photosensitivity is a well-documented phenomenon. Exogenous chemicals and drugs may cause photosensitivity by two main mechanisms: phototoxicity and photoallergy. Both processes occur as a result of an offending exogenous agent combined with light exposure. Multiple chemicals, such as psoralens, fluorescin dye, some thiazide diuretics, and some fluorquinolones are able to produce both types of cutaneous reactions. It may be difficult to distinguish between these entities; however, they are pathophysiologically distinct processes.

Phototoxicity is much more frequently encountered. It is typically an acute (within minutes to hours), chemically induced nonimmunologic skin irritation requiring light (photoirritation), which is prominent in areas of sun exposure and chemically resembles an exaggerated sunburn. Edema, pruritus, erythema, increased skin exposure, vesiculation, and desquamation may be present. These signs may be followed by long-lasting hyperpigmentation. In the classic form, a large amount of chemical or drug exposure is necessary to induce a phototoxic reaction. Histamine, kinins, and arachidonic acid derivatives, such as prostaglandins are released during the inflammatory processes. Histologic changes resemble those that would be seen in sunburned skin with epidermal dyskeratosis and vacuolation, as well as dermal edema and vascular changes. Mononuclear infiltrate may be evident.

Photoallergic reactions are much rare. In contrast to phototoxic reactions, photoallergies usually appear between 24 and 72 hours after exposure to a small amount of the exogenous chemical. Cutaneous manifestations resemble acute, subacute, or chronic dermatitis with significant pruritus, and the affected areas may be spread beyond areas of sun exposure. Photoallergy requires previous sensitization to the agent and is believed to be immune mediated. Reactions may result from cross-reaction between related chemicals. After drug cessation, re-exposure to the allergen may cause a reoccurrence of the reaction. This phenomenon does not occur with phototoxic agents. Histologic changes include epidermal spongiosis, perivascular lymphoidosis, and mononuclear exocytosis, which may resemble allergic contact dermatitis.

Clinical identification with photosensitivity reactions requires knowledge about skin effects of photosensitizing chemicals and clinical insight gained from practical experience. However, classic morphologic aspects of photosensitivity are not always apparent; prompt and accurate identification of phototoxic and photoallergic dermatoses induced by oral agents may be a challenge to the clinician.

PHOTOSENSITIZING AGENTS

Naturally occurring plant-derived furocoumarins, including psoralen, 5-methoxypsoralen (bergapten), 8-methoxypsoralen (xanthotoxin), angelicin, and others, constitute an important class of phototoxic chemicals. Bergapten, psoralen, and xanthotoxin are among the more commonly encountered phototoxic agents.

Psoralens are naturally occurring and are synthesized by plants of the Rutaceae (common rue, gas plant, Persian limes, bergamot) and Umbelliferae (fennel, dill, wild carrot, cow parsnip) (1). They also occur in a wild variety of other plants, such as parsley, celery, and citrus fruits (1,2). Phototoxicity reactions have been reported to psoralen-containing sweet oranges (3) and to the common rue (*Ruta graviolens*) (4).

Bergapten is the active compound of bergamot oil and is a well-known perfume ingredient whose toxic effects on the skin have been accorded the name berlock dermatitis. Based on the results of their studies of perfume phototoxicity, Marzulli and Maibach (5) suggested that perfume should contain no more than 0.3% bergamot, which is equivalent to about 0.001% bergapten, to avoid phototoxicity. Their work also established that bergapten was the only one of five components isolated from the oil of bergamot that was responsible for the phototoxic effects of the parent material. Limettin (5,7-dimethoxycoumarin), although more intensely fluorescent than bergapten, did not prove phototoxic to human skin. Bergapten phototoxicity continues to occur in some countries where bergapten-free bergamot is not used (6) in Norway from contact with *Heraculeum lacinatum* (7) and in Denmark from skin contact with *Heracleum mantegaz-zianum*, the giant hogweed (8).

Xanthotoxin (8-MOP) is effective in treating vitiligo and psoriasis by oral administration or topical application followed by exposure to ultraviolet-A (UVA) psoralen plus UVA light (PUVA phototherapy). The *Ammi majus* plant, containing xanthotoxin (8-MOP) in crude form, has been used therapeutically in Egypt since ancient times (9). However, at present, PUVA therapy is considered to have carcinogenic potential and warrants caution. Chronic use of this therapeutic regimen enhances prospects of inducing squamous cell skin cancer, especially in young patients and in those who are genetically predisposed (10). This potential has resulted in a reduced use of PUVA phototherapy in the United States (11).

Photochemical tissue bonding of skin (PTB) is a method of therapy using photochemical crosslinking of wound surface proteins via a photosensitizing dye [Rose Bengal (RB)] and green light (12). PTB is a light-activated technique that seals incisions in tissues, including skin, cornea, peripheral nerve, blood vessels, and

tendons, by applying a photosensitizing dye to the wound walls and then treating the skin surface with green (532 nm) light. The dye absorbs the light energy and initiates photochemical reactions leading to covalent proteins crosslinks that bridge the wound surface. The dye used for PTB, RB, is known to cause phototoxicity in cultured cells (13), yet the absorption of green light by RB does not show adequate significance for phototoxicity. Experiments in vivo (rabbit skin) and ex vivo (porcine skin) have shown that the RB concentration used with PRB for strong superficial wound closure does not cause phototoxicity (12). The phototoxicity of RB used in photodynamic therapy (PTD), however, has been shown to induce such cytogenic effects due to the reactive singlet oxygen use and the photochemical reactions within cells (PTB is occurs extracellularly).

There are a number of agents outside furocoumarin family that are phototoxic. Coal tar derivatives produce occupational contact photodermatitis and phototoxicity in industrial-based workers and road workers. Anthraquinone-based disperse blue 35 dye caused such effects in dye process workers. Radiation in the visible spectrum activates the dye (14). Pyrene, anthracene, and fluoranthene are strongly phototoxic to guinea pigs (15).

Phenothiazines, such as chlorpromazine, cause phototoxic effects, which have also been seen with oral therapeutic use of amiodarone, a cardiac antiarrythmic drug (16). Incidence, time course, and recovery from phototoxic effects of amidarone in humans were studied by Rappersberger et al. (17). Antimalarials quinine and quinidine appear to be phototoxic and some of these have been studied in vitro and in vivo (18–20). Cadmium sulfide, used in tattoos for its yellow color, may be phototoxic (21). Thiazide diuretics were shown to have a phototoxic potential in one study (22) but thiazide-induced phototoxicity is actually rare in clinical practice. There have been recent reports of phototoxicity induced by perforatum hypericum, contained in herbal antidepressant St. John's wort (23). This agent may function through mechanisms including inhibition of proteasome function (24).

Tetracyclines, particularly demethylchlortetracycline, and also doxycycline, chlortetracycline, and tetracycline, are phototoxic when orally ingested (25–27). Doxycycline was reported more potent than demethylchlortetracycline or limecycline in one human study (28).

Some fibric acid derivatives, such as fenofibrate, have been reported to exhibit photosensitizing effects in vivo. Bezafibrate and gemfibrozil are mildly phototoxic, and clofibrate has actually shown not to be phototoxic at all (29). Diltiazem has also shown to cause phototoxicity in some case reports (30).

Fluoroquinolone antibiotics have recently been proved to be phototoxic (31). There have been a number of controlled trials supporting this phenomenon. Fluoroquinolones differ significantly in their extent of phototoxicity. Recently, in a randomized, placebo-controlled study comparing phototoxicity, Dawe et al. (32) found sitafloxacin to be mildly phototoxic; enoxacin and sparafloxacin to be much more photoactive in white subjects. Levofloxacin and placebo failed to show a phototoxic effect. In contrast, among Asian subjects, sitafloxacin failed to demonstrate significant phototoxicity. A randomized-controlled trial supported the fact that lemofloxacin, but not moxifloxacin had phototoxic effects (33).

It is generally accepted that clinafloxacin > lomefloxacin, sparfloxacin > trovafloxacin, nalidixic acid, ofloxacin, ciprofloxacin > enoxacin, norfloxacin (34). Perfloxacin and sparfloxacin also appear to result in higher amounts of phototoxicity than ciprofloxacin (35). It is generally believed that levofloxacin and moxifloxacin are among the least phototoxic drugs in this class.

Antimicrobials, such as sulfonamides, and some fluoroquinolones (enoxacin and lomefloxacin) cause a cutaneous photoallergic reaction, as can sunscreen ingredients, most notably para-aminobenzoic acid and its derivatives, and fragrances, such as musk ambrette. As previously mentioned, thazides, fluorescein dye, and psoralens are phototoxic, as well as photoallergic.

Multiple case reports suggest that pyridoxine hydrochloride (Vitamin B6) may have some photoallergic activity and have been photopatch tested as positive for this agent (36,37).

Several psychiatric medications, including tricyclics, carbamazepine, and benzodiazepines, have shown to be cutaneous photoallergens.

Other miscellaneous drugs implicated in as photoallergens include amantidine, dapsone, nifedipine, and isotretinoin. However, for a number of these agents, formal data providing their photoallergenic potential are lacking.

NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a heterogenous group of compounds that exhibit favorable anti-inflammatory, analgesic, and antipyretic properties (38). NSAIDs were the subject of extensive investigations for phototoxic potential following reports that benoxaprofen, a suspended British antirheumatic NSAID, has this capacity (39–41). In vitro studies with sheep erythrocytes or human leukocytes suggested a phototoxic potential (41,42).

Propionic acids, specifically ibuprofen (IBU), are available for over-the-counter (OTC) sale. Because of its low pKa value (4.91), IBU exists predominately in its deprotonated or acidic form at physiologic pH. Phototoxicity has been a debated issue with IBU and other NSAIDs when human skin reacts abnormally to UV radiation or visible light (38). Studies of the phototoxicity of IBU have revealed that, despite its relatively high photostability (especially in comparison with its closely related ketoprofen), the formation of several products from decarboxylation can cause precursor situations to phototoxicity. However, computational data explains IBU's proposed photostability due to its low reactivity of the excited singlet state (38).

NSAIDs that are structurally related to propionic acid have been shown to possess phototoxic potential, whereas certain other types of NSAIDs, such as tenoxicam and piroxicam, were not experimentally phototoxic by in vivo or in vitro test methods (41,43,44). The propionic acid-derived NSAIDs produce unique immediate wheal and flare and are, in contrast, with a much delayed exaggerated sunburn response that typifies psoralen phototoxicity.

Although piroxicam is not phototoxic under experimental conditions, involving human test conditions (43), it has been implicated as a possible clinical photoallergic or phototoxic photosensitizer. One explanation for the unexpected photoactivity of piroxicam in skin is that a metabolite of piroxicam is indeed phototoxic when isolated and tested on human mononuclear cells in vitro (44). These positive findings and likely explanation are related to the production of singlet oxygen, as indicated by emission at 1270 nm when the subject metabolite was irradiated with UV in vitro (44,45).

Other propionic acid-derived NSAIDs associated with an immediate phototoxic response are nabumetone (MNAA), naproxen (NP), and tiaprofenic acid (43,46). When taking into account that most of the phototoxic action occurs after decarboxylation of NP versus MNAA, NP is concluded to be more phototoxic than MNAA (47).

Carprofen (48), ketoprofen (49), benzydamine hydrochloride, topical tiaprofenic acid, suprofen, and possibly piroxicam appear to be photoallergenic. However, further work may be possibly needed to separate, clarify, and identify three possible outcomes—allergy, photoallergy, and phototoxicity—in studies involving NSAIDs.

The general area of cutaneous reactions to NSAIDs has been extensively reviewed by Ophaswongse and Maibach (50).

MECHANISMS OF PHOTOTOXICITY

Phototoxicity and photoallergic chemicals typically exhibit biologic response with the UV area of the electromagnetic spectrum, which is subdivided arbitrarily into UVA (320–400 nm), UVB (290–320 nm), and UVC (200–290 nm). UVA represents the less energetic portion of the spectrum and UVC the more energetic (cytotoxic) area. UVA in the range 320–340 nm (UVA2) is more energetic and more skin damaging than UVA in the range 340–400 nm (UVA I). In vivo, both phototoxicity and photosensitivity are primarily due to UVA range light. However, in vitro, phototoxic agents absorb and are activated by both UVA and UVB wavelengths. The cause of this discrepancy is unknown. Some phototoxic chemicals, such as porphyrins and fluorescein dye, absorb visible light (400–800 nm).

Exogenous phototoxic reactions are initiated when a photoactive chemical (one capable of absorbing UV radiation) or one of its metabolites enter viable skin cells. The photoactive chemical may enter into the skin by topical administration or it may reach the skin indirectly by the circulatory system following ingestion or parental administration. Some systemically administered and possibly topical chemicals may require conversion to become photoactive. When the photoactive chemical is in the skin, appropriate wavelengths of light penetrate the skin and subsequently photons are absorbed by, and thereby excite electrons in the phototoxic chemical. This process may lead to the formation of unstable singlet or triplet states. Additionally, the singlet UV-excited state can react and once the triplet state is formed, it will react with another oxygen molecule to create a singlet oxygen concomitant with relaxation of the triplet state into the initial excited state. This re-cyclization process continuously generates a singlet oxygen species that is a starting process for phototoxicity (47). As these molecules transfer energy to achieve a more stable state, the transferred energy induces cellular damage and generates inflammatory mediators.

The questions of site and mechanism of action of phototoxic chemicals and the importance of oxygen have been much studied. Some phototoxic agents are oxygen dependent, or photodynamic, whereas others are not. Photodynamic chemicals may transfer their energy to oxygen, exciting it to the singlet or doublet state, thereby exerting phototoxic effects. In its excited state, the photodynamic chemical may react with oxygen and form free radicals. Although mechanisms causing reactions of photoactive drugs are mainly free radical in nature, reactive species of oxygen are also involved. Photochemical activity of drugs, such as hydrochlorthiazide, furosemide, chlorpromazine, and some NSAIDs is caused by free radical formation. In other systems, the reactive excited singlet form of oxygen is directly toxic toward lipids and proteins (51). Singlet oxygen, superoxide radical anions, and peroxylic radical species are expected to be formed in different steps throughout the photodegradation of NSAIDs NP and the active MNAA, which subsequently will produce their action of biomolecules, including the initiation of propagation lipid peroxidation reactions (47).

Studies by Gendimenico and Kochevar (52) have shown that acridine requires oxygen to produce lethal (phototoxic) effect on mast cells. (Dermal mast cells are known to participate in cutaneous phototoxic responses initiated by UV and visible radiation.) Chlorpromazine is also thought to be activated by a photodynamic process involving molecular oxygen.

Reactive oxygen intermediates may be a main cause of photosensitivity reactions, which can be stopped by agents that block the production of these intermediate products. Antioxidant supplementation may be beneficial in suppressing phototoxic reactions. Vasoactive amines, such as antihistamine and serotonin, may also play a role in cutaneous phototoxic reactions. Eucosinoids, such as prostaglandins and leukotrienes, have also been implicated in the process.

Mathews (53) showed that toluidine blue requires oxygen to produce its lethal (phototoxic) effect on *Sarcina lutea*; however, oxygen is not needed for the phototoxic effect of 8-MOP on *S. lutea*. In addition, it was found that 8-MOP phototoxicity results in damage to cellular DNA, whereas toluidine kills by action on the cell membrane. Psoralens also do not require molecular oxygen to produce phototoxic effects.

Some photoactive chemicals act on cellular DNA (psoralens, may be tricyclics), whereas others act on cellular membranes (tricyclics). Fluoroquinolones may induce DNA breaks and lead to cell death. Keratinocytes may be the most sensitive and melanocytes the most resistant (54). The difference in phototoxicity potential may be based on the differences in substituent placement on the various chemicals (55).

Photoallergic reactions are believed to be cell mediated, with radiation-dependent antigen production, therefore stimulating the immune response. UV energy may cause the drug hapten to find a native protein on epidermal cells, therefore forming a complete photoantigen. When the antigen is formed, the photoallergic process is similar to allergic contact dermatitis, with sensitization of the immune system, and a subsequent cutaneous eruption.

A more complete discussion of mechanisms of photosensitivity reactions is given by Spikes (56).

ELEMENTS OF THE TEST FOR PHOTOTOXICITY

Tests for phototoxic potential of topically applied chemicals are usually conducted with radiation within the UVA range. Some phototoxic chemicals are activated by wavelengths in the visible spectrum (bikini dermatitis) (57), some by UVB (58), and some (doxycycline) are augmented by UVB (59).

Accurate measurements of radiation intensity and frequency are important prerequisites for work in phototoxicity.

Phototesting procedures include photopatch testing and determination of minimal erythema dose (MED) for UVA and UVB. Photopatch testing may be more useful in detecting photoallergy, and MED may be more useful for testing phototoxic agents. In practice; however, it is recommended to perform both types of testing to ensure comprehensive evaluation.

Among animal models for which photopatch testing has proved to be useful in predicting human phototoxicity are the mouse, rabbit, swine, guinea pig, squirrel monkey, and hamster, in that approximate order of effectiveness (5).

The test material is applied to the skin of a human subject or an animal model (clipped skin of mouse, guinea pig, rabbit, or swine). After a suitable waiting period for skin absorption to take place (several minutes, depending on the rate of skin penetration), the chemical test site is irradiated with UV of appropriate wavelengths. The test site is then examined at 1, 24, 28, and 72 hours for evidence of phototoxicity, such as erythema, vesiculation, bullae, and finally, hyperpigmentation. A comparison is made between the skin of test site and control sites (one without chemical and one without light).

Results are modified by factors that affect skin penetration, such as test concentration and vehicle, as well as by duration of exposure and by distance from irradiation source to the test area.

Some photoirritants (e.g., bergapten), produce clinical phototoxicity when the photoirritant site is irradiated within minutes to 1 hour after skin application; with others, irradiation is effective when administered at 24 hours.

Phototoxic effects are expected when UV is directed and absorbed by a phototoxic chemical residing in the skin. This results in a skin reaction with cellular components, such as DNA.

One of the earliest indicators of phototoxic potential was based on a paralyzing effect on the cilia of Paramecium from acridine plus light, reported by Oscar Raab at the close of the nineteenth century. This test method was later followed by a simpler test involving a lytic effect on red blood cells, as an endpoint of phototoxicity.

The subject of in vitro assay for phototoxic effects has recently been reviewed (60).

Recently, reconstructed human epidermis models, such as EpiSkin, ShineEthine, and EpiDerm, have demonstrated the ability to serve as in vitro models for phototoxicity testing. Certain models have proved to be effective in discriminating between phototoxic and nonphototoxic compounds compared with the in vivo data. Several protocols for use are currently available. In the future, data obtained from these models will probably contribute a wealth of information, thereby increasing our knowledge and understanding of photosensitivity.

HIGHLIGHTS

Investigative studies in photosensitivity require a rudimentary understanding on what constitutes radiation sources for experimental work, as a first step. Knowledge about safety in the use of radiation equipment is equally important.

Well-calibrated equipment for measuring radiation is another prerequisite, including recognition that with time and use, equipment changes and requires proper upkeep to ensure its quality performance.

Filters are sometimes needed to provide an appropriate cutoff of unwanted radiation. Window glass is useful in eliminating wavelengths below 320 nm.

Natural sunlight is filtered by atmospheric oxygen, ozone, clouds, particulates, and other environmental factors, including altitude, so that wavelengths below 290 nm are effectively shielded from reaching the earth's surface. Consequently, radiation sources that deliver highly energetic shorter wavelengths are unlikely to be useful in experimental photosensitivity studies involving humans.

The radiation ranges that are of the greatest biologic focus in photosensitivity studies are UVA (320–400 nm), UVB (280–320 nm), and UVC (<280 nm). As the Commission de l'Eclairage recommends 315 nm as the cutoff for UVB rather than 320 nm, it is important that the investigative photobiologist identifies the system of use. However, a rationale for using 320 nm rather than 315 nm as the cutoff for UVA is given by Peak and van der Leun (61).

TABLE 13.1
Chemicals, Plants, and Drugs with Phototoxic Potential

Topical dyes: anthraquinone, fluorescein dye, disperse blue 35, cosin, methylene blue, Rose Bengal, toluidine blue, cadmium sulfide in tattoo
Fragrances: oil of bergamot
Flurocoumarins: angelicin, bergapten, psoralen, 8-methoxypsoralen, 4,5, 8-trimethylpsoralen
Plant products: celery, fig, lime, hogweed, parsnips, fennel dill
Coal tar components: acridine, anthracene, benzopyrene, creosote, phenanthrene, pitch, pyridine
Systemic antibiotics: griseofulvin, ketoconazole, nalidixic acid, sulfonamides, ceftazidime, tetracyclines, fluoroquinolones
Chemotherapeutics: dacarbazine, 5-fluorouracil, vinblastine, methotrexate
Drugs: amiodarone, chlorpromazine, quinine, quinidine, tolbutamide, diltizem, fibric acid derivatives, hyperpicum perforatum (St. John's wort)
Diuretics: hydrochlorothizidine, bendroflumethiazide, furosemide
Nonsteroidal anti-inflammatories: benoxaprofen, naproxen, piroxicam, tiaprofenic acid, nabmetrone
Porphyrins: hematoporphyrin

The first rule of photochemistry is that cells are injured or killed when photons of radiant energy are absorbed and energy is transferred to target molecules (56). Phototoxic effects are therefore produced when absorption wavelengths of the sensitizer are the same as those of the radiant energy source (Grotthus–Draper law).

DNA, RNA, deoxy- or ribodeoxynucleotides, enzymes containing such cofactors, and aromatic and cysteine residues of proteins are typical targets of UV phototoxic damage.

Oxygen may or may not participate in the production of a phototoxic event; however, when oxygen is indeed involved, it is often referred to as a photodynamic action.

Psoralens are among the most frequently encountered phototoxic chemicals, as they are present in many plants. Petroleum products, coal tar, cadmium sulfide, acridines, porphyrins, and other chemicals may also be implicated as causative agents for phototoxic effects. Table 13.1 provides a list of phototoxic chemicals.

Finally, it is suggested that investigators be complete in identifying equipment and methodology that they employ to reduce some of the confusion that may enter and has already entered the literature on this subject.

CONCLUSIONS

Years of investigative efforts, along with improved methods of measuring and administering radiation, have brought considerable progress in our understanding of various aspects of photosensitivity. We appear to have identified and continue to identify major chemical structures that are currently involved in producing phototoxic and photoallergic effects in humans. We have also gained some insight into some of the mechanisms that are involved. Nevertheless, it is always important to be flexible and aware that time may change some of our present and apparently well-conceived perceptions, as it often does.

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14 Contact urticaria syndrome

Ana Gimenez-Arnau

The contact urticaria syndrome (CUS) comprises a heterogenous group of immediate contact inflammatory reactions that usually appear within minutes after contact with eliciting substances. Occasionally systemic involvement can be present. It was defined as an entity in 1975 by Maibach and Johnson (1). Since then and nowadays its scientific interest increased and new cases are continuously reported providing information concerning new trigger factors and clinical features.

Contact urticaria (CoU) refers to a wheal and flare reaction following external contact with a substance, usually appearing within 30 minutes and clearing completely within hours, without residual signs (2). The term was introduced by Fisher (1973), but this phenomenon has long been recognized (3). Urticaria lesions to nettles and hairy caterpillars were reported in the 19th century and continue being reported today (4). In a recent randomly designed survey carried out in 1224 adults in Spain, contact wheals and pruritus were noticed by the 52.1% and 100%, respectively, of people who suffered cutaneous symptoms induced by pine processionary (5). Furthermore, some naturally existing urticariogens were used therapeutically as rubefacients, counter-irritants, and also vesicants (6).

Hjorth and Roed-Petersen (1976) defined protein contact dermatitis (PCD) as characterizing an immediate dermatitis induced after contact with proteins (7–9). Thirty-three food caterers suffering exacerbation of the itch, immediately after contact with meat, fish, and vegetables followed by erythema and vesicles were described. The application of relevant foods to the affected skin resulted in either urticaria or eczema (10). Atopy and PCD are associated in approximately 50% of affected patients (11).

Patients with CUS can develop immediately after the contact with the trigger substance, CoU, and/or dermatitis/eczema. These immediate contact reactions appear on normal or eczematous skin. Wheals are the characteristic symptoms in CoU. Eczema appears rapidly on the hands in PCD. Both cutaneous symptoms and entities can be induced by the same trigger factor and can be suffered by the same patient.

HOW FREQUENT IS AND WHICH SOCIAL IMPACT HAS CONTACT URTICARIA SYNDROME

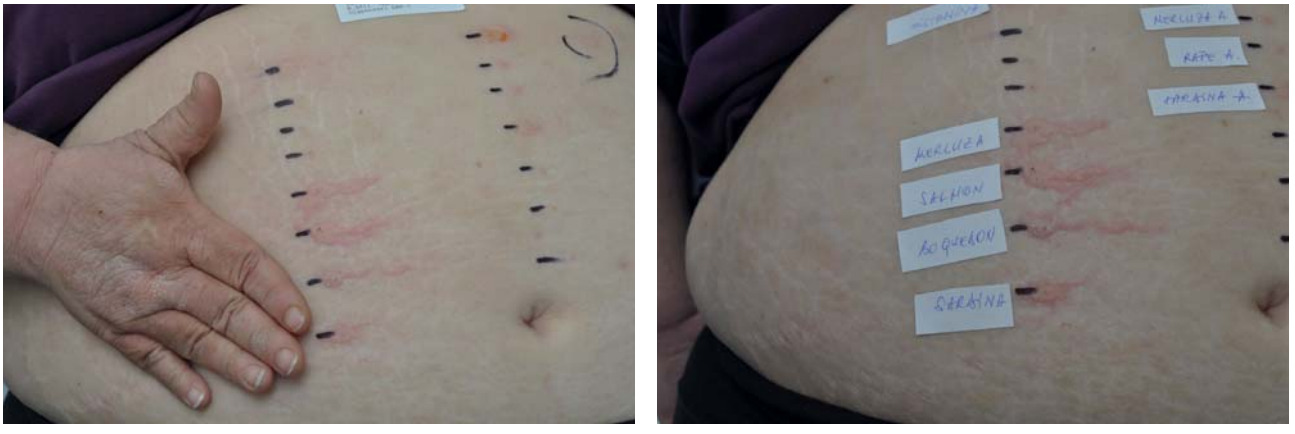
The global incidence of CUS is not known but immediate contact reactions are common in dermatologic practice (12–17). With the exception of latex allergy showing prevalence of 5–10%, for the rest of the trigger factors just isolated cases or short series of patients are described (18). In the occupational setting, CUS seems to be common although a precise statistical analyses is difficult to obtain in most of the countries because of under-reporting (19). In a few countries, CoU has been classified as a separate occupational skin disease. This is the case in Finland since 1989. The "Finnish Register of Occupational Diseases" (1990–1994) showed that CoU was the second most frequent cause of occupational dermatosis (29.5%), after contact allergic dermatitis (70.5%) (20,21). The trigger agents were cow dander (44.4%), natural rubber latex (23.7%), and flour, grains, or feed (11.3%) (21). Less proportion of occupational CoU was found in a retrospective study done in a tertiary level clinic specializing in occupational dermatology in Melbourne, Australia, showing an 8.3% CoU prevalence (22). Hands, arms, and face were the most frequent body area involved. Atopy was a significant risk factor for natural rubber latex, foodstuffs, or ammonium persulfate CoU. Health workers, food handlers, and hairdressers were the most common occupations affected. More recently, a survey conducted in 335 restaurant, catering, and fast-food employees in Singapore showed as more common occupational dermatosis irritant contact dermatitis (10%) being occupational CoU urticaria sporadically reported in only two patients as caused by lobster and prawn (23). The nature of the exposure will probably determine the percentage of CoU risk.

Occupational screening questionnaires including specific questions searching for urticaria symptoms are very few. The long version of the Nordic Occupational Skin Questionnaire (NOSQ-2002) is one of them including 9 questions about urticaria symptoms (24). A standardized method to evaluate the occupational relevance of CoU, such as that already developed for occupational contact dermatitis, Mathias criteria (25), would be desirable.

Health care workers in Europe show a known prevalence of occupational CoU from 5 to 10%, whereas in the general population it lies between 1 and 3%. People of other occupations also have a high risk for developing CoU as there are food handlers or people involved in agriculture, farming, floriculture, plastics, pharmaceutical and other laboratories, as well as hunters, veterinarians, biologists, or hairdressers. Atopy favors further sensitization where protein allergens are concerned (26).

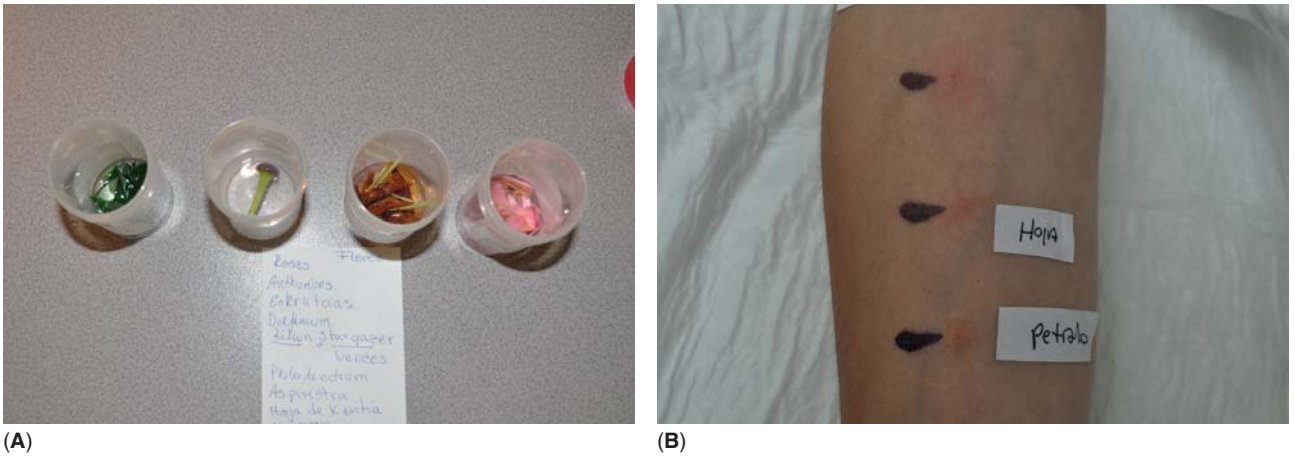
HOW CONTACT URTICARIA SYNDROME IS CLINICALLY MANIFESTED

The CUS has been classified into four stages. The stages 1 and 2 show cutaneous symptoms. Stage 1 includes flare reactions, wheals, and eczema as well as symptoms, such as itching, tingling, or burning sensation. When CoU is present it shows itchy wheals, which are usually strictly limited to contact areas and which disappear within a few hours without residual lesions. PCD typically affects the hands (especially the fingertips) and sometimes extends to the wrists and arms (Fig. 14.1). Chronic paronychia with redness and swelling of the proximal nail fold after handling food (27) and natural rubber latex (28) can also be



(A) (B)

FIGURE 14.1 **(A, B)** Eczema at the dorsum of hand induced by proteins habitually touched in the daily work of a fisher woman sailor. Positive wheal induced by prick by prick test with hake, salmon, anchovy, and sardine.



(A) (B)

FIGURE 14.2 **(A, B)** Positive prick by prick test to leaves and petals from Lilium Stargazer in a young women who suffered contact urticaria with systemic involvement and anaphylaxis in presence and touching this ornamental flower.

observed in PCD. Stage 2 refers to the development of generalized urticaria after a local contact. Stages 3 and 4 include extracutaneous reactions or symptoms that may also occur as part of a more severe reaction. Stage 3 may include bronchial asthma, rhinoconjuntivitis, orolaryngeal symptoms, or gastrointestinal dysfunctions. Internal organs may be involved in CUS patients, depending on the allergen or pre-existing conditions, such as atopic dermatitis (29,30). By contact or in the case of a volatile allergen, rhinoconjuntivitis and asthma may accompany the skin manifestations, as occurs with bakers who are in continuous contact with flour. Abdominal pain, diarrhea, and oral allergy syndrome may develop when the allergen comes in contact with the oropharyngeal mucosa (31). The severity of this multisystemic disease has been reported by Von Krogh and Maibach (32). Finally, in stage 4, anaphylactic or anaphylactoid reactions may occur as the most severe type of CUS manifestation (Fig. 14.2), Contact urticaria can be life threatening—certain substances, such as latex protein, can induce anaphylaxia and even death.

The oral allergy syndrome (OAS) can be considered as a special form of CUS localized in mouth and throat. Usually its symptoms are immediate after oral contact with the food involved. They include oralpharyngeal pruritus (itching of mouth, palate, and throat), angioedema of lips, tongue and palate, and hoarseness. The oral syndrome can be accompanied by gastrointestinal reactions and systemic involvement showing urticaria, rhinitis, asthma, or even anaphylaxis.

WHAT WE KNOW ABOUT THE MECHANISMS INVOLVED IN CONTACT URTICARIA SYNDROME

The mechanisms underlying immediate contact skin reactions are partially understood. Each trigger substance has its own mechanism or mechanisms of action. Nonimmunologic CoU (NICoU) is due to vasogenic mediators without the involvement of immunologic processes. Urticariogens may act following different patterns. The most classic example concerns dimethyl sulfoxide (DMSO), which damages the blood vessels, making them leaky and inducing mast cell degranulation (33). Antihistamines do not inhibit reactions to DMSO and other NICoU-responsible agents, but acetylsalicylic acid and nonsteroidal anti-inflammatory drugs do (both orally and topically); therefore, a role of prostaglandins has been suggested (34–36). The release of prostaglandin D2 without concomitant histamine release has been demonstrated after topical application of sorbic acid and benzoic acid (37,38). Capsaicin pretreatment (which depletes substance P) does not impair NICoU, but does inhibit the allergen prick test flare of

immunologic CoU (ICoU) (39). Nonspecific tachyphylaxis of variable duration has been associated with various urticariogens (40). Sharp hairs from animals or spines from plants penetrating the skin can deliver a cocktail of irritant chemicals or proinflammatory mediators causing NICoU (41).

The pathogenesis of immunologic CoU (ICoU) reflects a type I hypersensitivity reaction, mediated by allergen-specific immunoglobin E (IgE) in a previously sensitized individual (42). Skin challenge involves allergen penetration through the epidermis, IgE binding on mast cells, its degranulation, and subsequent release of histamine and other vasoactive substances as prostaglandins, leukotrienes, and kinins.

The OAS is generally due to an IgE-mediated type I allergic response. People with birch pollinosis show cross reactivity because its structural homology with Rosaceae fruits as apple or peach (43–45). Nevertheless, some other foods as peanut (Ara h1 and 2) or fruits can induce OAS independently of pollinosis.

A combination of type I and type IV allergic skin reactions, the latter supported by positive delayed patch tests, has been suggested as PCD pathogenesis (46,47). It has been speculated that PCD is an eczematous IgE-mediated reaction through proteins. PCD shows a similar reaction pattern to aeroallergen-induced atopic eczema or dermatitis (48).

DIAGNOSTIC TOOLS USEFUL TO MAKE AN ETIOLOGIC DIAGNOSIS

Diagnosis of CUS is based on full medical history and skin testing with suspected substances (Fig. 14.3). In vitro techniques are available for only a few allergens, including latex. The simplest cutaneous provocation test for ICoU, NICoU, and immediate contact dermatitis as PCD is the "open test." The suspected substance is applied and gently rubbed on slightly affected skin or on a normal-looking 3×3 cm area of the skin, either on the upper back or the extensor side of the upper arm. Often it is desirable to apply contact urticants to skin sites suggested by the patient's history. A positive result is an edema and/or erythema typical of CoU, or tiny intraepidermal spongiotic vesicles typical of acute eczema. Immunologic and nonimmunologic contact reaction usually appears within 15–20 minutes being the nonimmunologic taking a little longer between 45 and 60 minutes. ICoU can also show a delayed onset, although this is rare. When the open test results are negative, "prick testing" of suspected allergens is often the method of choice for immediate contact reactions. "Scratch test" and "chamber scratch test" (contact with a small aluminum chamber for 15 minutes) are less standardized than the prick test, but are useful when a nonstandard allergen must be studied. For both prick and scratch tests, histamine hydrochloride serves as the positive control and aqueous sodium hydroxide as negative reference. When other than cutaneous organs are involved, it is important to begin ICoU testing with much diluted allergen concentrations and to use serial dilutions to minimize allergen exposure. When testing with poorly or nonstandardized substances, control tests should be assessed on at least 20 people to avoid false-positive interpretation. Nonsteroidal anti-inflammatory drugs and antihistamines should be avoided because of the risk of false-negative results. Following the recommended protocol is important for minimizing the occurrence of hazardous extracutaneous reactions. Life-threatening reactions have been documented during skin tests; therefore, caution is advised, especially when testing certain occupational substances. Skin tests should be performed only if resuscitation equipment and trained personnel are readily available (49–51).

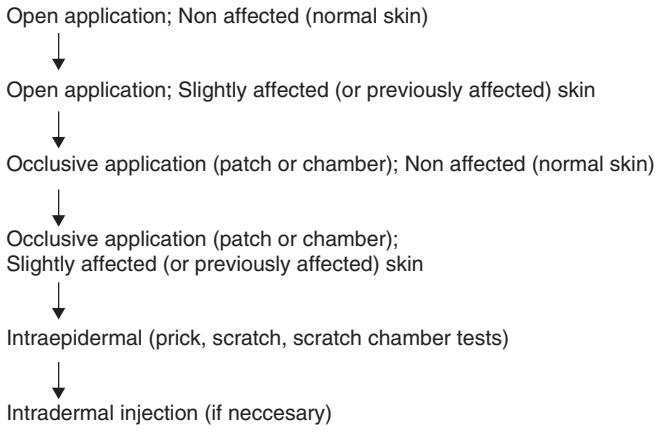


FIGURE 14.3 Diagnostic algorithm to test contact urticaria and protein contact dermatitis from the contact urticaria syndrome.

DEMONSTRATED RESPONSIBLE AGENTS IN CONTACT URTICARIA SYNDROME

Proteins (molecular weight 10,000 kDa to several hundred thousands) and also chemicals (molecular weights below 1000 kDa) can trigger CUS (52).

Plant or animal proteins; chemicals, such as drugs and preservatives; or more diverse substances, such as metals and industrial chemicals can induce ICoU. Raw fruits and vegetables are a common cause of ICoU in daily life. Natural rubber latex allergy focused global interest at the end of the 20th century. Latex sensitization risk factors include atopy and prolonged exposure via damaged epidermis, for example, glove wearers with hand eczema. Low–molecular weight molecules normally act as haptens; nevertheless, for some of them IgE antibodies have been also demonstrated as, for example, sensitized workers reactive to platinum and nickel–serum albumin complexes (53,54).

NICoU is defined by stinging nettles wheals induced from *Urtica dioica*. Other responsible agents are preservatives, fragrances, and flavorings in cosmetics, toiletries, topical medications, or foodstuffs as benzoic and sorbic acid (55) Household, industrial, insecticide, and laboratory chemicals can also induce NICoU.

Few substances elicit mixed features of NICoU and ICoU through an unestablished mechanism. Other than the above IgE is involved in ammonium persulfate-induced CoU, where specific IgG and IgM activate the complement cascade through the classical pathway (56–58). Immediate reactions to formaldehyde seem not to be mediated by IgE being a prostaglandin role suspected because of thromboxane B_2 and prostaglandin PGF_2 increase levels (59,60).

A huge amount of compounds can be responsible for occupational and nonoccupational CUS, including animal products, plants and plant derivatives, foods, fragrances, cosmetics, flavorings, medications, preservatives, disinfectants, enzymes, metals, and miscellanea of different substances. Tables 14.1–14.6 include most of the compounds whose responsibility has been registered in the literature (61–121).

TABLE 14.1
Animals, Plants and Derivatives (Natural Products) Responsible for Immediate Contact Reaction

Animal, Plants and its Derivatives

- Amniotic fluid[a,b]
- Anisakis simplex
- Blood[a,b]
- *Brucella abortus*[b]
- Calf[a]
- Cow[a]
- Caterpillars[c]
- Cephalopods (*Loligo vulgaris*)[a]
- Chironomus[b]
- Cockroaches[a,b]
- Corals[c]
- Dander[a,b]
- Dogs; milk, seminal fluid[a]
- Gut (pig)[a,b]
- Guinea pig[a,b]
- Hair (rat, mice)[a,b]
- Horse
- Human hair
- Jellyfish[b,c]
- Liver (mouse)[a]
- Locust Tee[a,b]
- *Lumbrinereis impatiens*
- Mites[a,b]
- Moths[c]
- *Nereis diversicolor*
- Pig[a]
- Pearl oysters[a]
- Placenta (cow)[a,b]
- Saliva[b]
- *Sarcophaga carnaria*
- Rat[a]
- Roe deer
- Seminal fluid[b]
- Serum (amphibian)[a,b]
- Silk[b]
- Spider mite[a,b]
- Urine (mice, rat)[a,b]
- Worms[a]
- Algae[b]
- Aloe (Morrow)
- Arugula[b]
- Birch[b]
- Boungainvillea
- Camolile[a]
- Chamomilla
- *Cannabis sativa*[a]
- Chrysanthemum[a,b]
- Cinchona[a]
- Coral[c]
- Corn powder
- Cotoneaster
- *Crateagus* (hawthorn)[b]
- Elm tree
- *Eruca sativa*[b]
- Eucalyptus[b]
- *Ficus benjamina*[b]
- Gerbera[a]
- *Grevillea juniperina*[c]
- *Hakea suaveolens*
- Larch
- Lichens
- Lilies[a,b]
- Lime (Tilia)
- *Limonium tartaricum*[a,b]
- Mahogany[a]
- Mulberry
- Obeche[a,b]
- *Phaseolus multiflorus*
- Parsley[b]
- Poppy flowers[b]
- Sea anemone[c]
- *Semecarpus anacardium*
- Sesame seeds[b]
- Sunflower seeds[b]
- Teak[b]
- Tobacco[a,b]
- Tropical woods[a,b]?
- Tulips[a,b]
- Verbena[a,b]

Plant derivatives

- Abietic acid
- Colophony[a,b]
- Cornstrach[a,b]
- Latex rubber[a,b]
- Turpentine[c]

[a]Occupational.[b]Immunologic.[c]Nonimmunologic.
Source: Adapted from Ref. 49.

TABLE 14.2
Foods and Food Additives Responsible for Immediate Contact Reaction

- Meat[b]
- Beef[a]
- Calf[a,b]
- Chicken[a]
- Codfish
- Ham (T, putrescentiae)
- Lamb
- Liver
- Pork[a]
- Sausage
- Turkey
- Fish[a,b,c]
- Cod[a]
- Crab
- Frog[a,b]
- Herring[a]
- Lobster[a]
- Lupin
- Oysters[a]
- Plaice[a]
- Pork[a]
- Raw fish[a]
- Seafood[b]
- Shrimp[a]
- Other animal products
- Cheese[a]
- Eggs[a]
- Honey
- Milk[a]
- Fruits[b]
- Almond[a]
- Apple[a]
- Apricot
- Apricot stone[a]
- Banana
- Kiwi
- Litchi
- Lemon[a]
- Lemon peel[a]
- Lime[a]
- Mango
- Nuts[b]
- Orange
- Peach
- Peanuts
- Peanut butter
- Plum
- Strawberry[a]
- Watermelon[a]
- Seeds[b]
- Sesame seeds[b]
- Sunflower seeds[b]
- Grains[b]
- Buckwheat[a]
- Flour[a]
- Maize[a]
- Malt
- Rice[a]
- Wheat[a]
- Wheat bran
- Vegetables[b]
- Asparagus[a,b]
- Arugula[b]
- Beans[a]
- Cabbage[a,b]
- Carrots[a]
- Castor bean[a,b]
- Celery[a]
- Chamomilla
- Chicori
- Chives
- Coffee been (green)[a,b]
- Cucumber pickle[a,b]?
- Dill[b]
- Endive[a,b]
- Fungi
- Garlic[a,b]
- Lettuce[a,b]
- Lime[a]
- Mentha[a]
- Mushrooms[a,b]
- Mustard[a,b]
- Onion[a,b]
- Parsley[a]
- Parsnip[a]
- Potato[a]
- Rice 7
- Rocket
- Runner bean[c]
- Rutabaga (Swede)
- Salami casing molds[a,b]
- Soybean[a]
- Stock (*Matthiola incana*)
- Tomato[a,b,c]
- Winged bean[a]
- Flavoring and fragances
- Balsam of Peru[b,c]
- Benzaldehyde[a,c]
- Benzoic acid
- Cinnamon oil
- Cinnamic acid[c]
- Cinnamic aldehyde[a,c]
- Gum arabic I[a]
- Menthol[c]
- Vanillin[c]
- Condiments and species
- Cayenne pepper[c]
- Caraway[a]
- Coriander
- Curry[a]
- Paprika (*Capsicum annuum*)[a,b]
- Thyme[c]
- Coloring agents
- Amaranth
- Allura red
- Cochineal red
- Ponceau
- Sunset yellow
- Tartrazine

[a]Occupational.[b]Immunologic.[c]Nonimmunologi.
Source: Adapted from Ref. 49.

TREATMENT AND PREVENTION OF CONTACT URTICARIA SYNDROME

CUS clinical symptoms are determined by the route, duration, and extent of exposure, the inherent sensitizing properties of the allergen, and an individual's genetic and/or acquired susceptibility. Discovering the responsible agent is required to identify the correct avoidance of the eliciting trigger. Avoidance of further exposure will improve occupational contact dermatitis and CoU. Primary and secondary prevention are highly recommended being necessary common guidelines to prevent well-known occupational risks as, for example, latex allergy (122).

Considering their good safety profile, second-generation antihistamines must be considered the preferred firstline symptomatic treatment for most of the CoU (123). Before considering alternative treatment, higher doses of antihistamines should be used. When dermatitis is present topical immunomodulation can be conducted using topical steroids. Severe cases of CUS require a short course of oral steroids or even treatment in an emergency unit.

TABLE 14.3
Fragances and Cosmetics Responsible for Immediate Contact Reaction

- Hair care products
  - Ammonium persulphate[a]
  - Basic blue 99 (amino ketone dye)[b]
  - Henna[a,b]
  - Panthenol
  - Protein hydrolysate[a]
  - Paraphenylenediamine[a,b]
- Emulsifiers
  - *Cetyl alcohol*
  - Polysorbate
  - Sorbitan monolaurate
  - Sorbitan monostearate
  - Sorbitan sesquiolate
  - Stearyl alcohol
- Fragances
  - α-amyl cinnamic aldehyde[c]
  - Anysil alcohol[c]
  - Balsam of Peru[a,b,c]?
  - Cassia oil[c]
  - Cinnamic aldehide[c]
  - Cinnamic alcohol[c]
  - Cinnamic acid[c]
  - Coumarin[c]
  - Eugenol[c]
  - Geraniol[c]
  - Hydroxycitronellal[c]

Other substances
- Allantoin
- Aloe gel[b]?
- Benzophenone[b,c]
- Chamomile extract[b]?
- Lecithin[b]?
- Melissa extract[b]?
- Pyrrolidone carboxylate[c]
- Propylene glycol[c]
- Resorcinol[c]
- Wheat[a,b]
- Wool alcohol[b]

[a]Occupational.[b]Immunologic.[c]Nonimmunologic.
Source: Adapted from Ref. 49.

TABLE 14.4
Drugs Responsible for Immediate Contact Reaction

- Acetylsalicilyc acid
- Aescin[b]?
- Aminophenazone
- Ampicilin[b]
- Amoxicilin[a]
- Bacitracin[b]
- Benzocaine
- Benzoyl peroxide[b]
- Capsaicin[c]
- Carboxymethylcellulose sodium[b]
- Chloroform[c]
- Cephalosporins[a,b]
- Cisplatin[a,b]
- Chloramphenicol[b]
- Chlorpromazine
- Dinitrochlorobenzene
- Diphenylcyclopropenone[b]
- Dimethylsulfoxide[c]
- Donezepil
- Gentamycin[b]
- Guanidinium salts[a]
- Hexylene glycol[b] (excipient)
- Iodochlorhydroxyquin[b]
- Ketoprofen
- Lidocaine
- Levopromazine[a]
- Lindane[b]
- Mechlorethamine[b]
- Methamizole[a]
- Mezlocillin[a,b]
- Neomycin[b]
- Nicotinic acid esters[c]
- Penicillin[a,b]
- Pentamidine isethionate[a,b]
- Phenothiazides[b]
- Pilocarpine
- Prophylphenazone
- Promethazine
- Pyrazolones[b]
- Rifamycin[b]
- Steroids
- Streptomycin[a,b]
- Tar extracts[c]
- Tincture of benzoin[c]
- Uranium salts[a]
- Virginiamycin[b]

[a]Occupational.[b]Immunologic.[c]Nonimmunologic.
Source: Adapted from Ref. 49.

TABLE 14.5
Preservatives Responsible for Immediate Contact Reaction

- Acetic acid
- Aescin polysulfate
- Alcohols[b,c]
  - Amyl
  - Ethyl
  - Butyl
  - Isopropyl
  - Benzyl[b,c]
- Ammonia[b]
- Benzoic acid[b,c]?
- Benzyl alcohol
- Bronoprol[c]
- Butilated hydroxytoluene[b]?
- Camphor[c]
- Chloramine[b]
- Chlorhexidine[b]
- Chlorine
- Chlorocresol[a,b,c]
- Formaldehyde[a,b,c]
- Gentian violet[b]
- Hexylene glycol[b]
- Imidazolidinyl urea[c]
- Kathon CG[c]
- Mercurochrome[b]
- α-Phenylphenate[b]
- P-chlorocresol
- Parabens[b]?
- 2-Phenoxyethanol
- Phenylmercuric acetate[a,b]
- Phenyl mercuric propionate[b]
- Polyethileneglycol
- Sodium benzoate[a,c]
- Sodium hypochlorite[b]
- Sorbic acid[c]

[a]Occupational.[b]Immunologic.[c]Nonimmunologic.
Source: Adapted from Ref. 49.

TABLE 14.6
Miscellaneous Chemicals and Metals Responsible for Immediate Contact Reaction

- Acetyl acetone[b]
- Acid anhidrides[a,b]
- Acrylic acid[b]?
- Acrylic monomers[a,b]
- Aliphatic polyamide[a,b]
- P-aminodiphenylamine[a,b]
- Aminothiazole
- Aziridine[a,b]
- Benzonitrile[a]
- Butylhidroxytoluol
- Calcium hypochloride
- Carbamates[a,b]
- Carbonless copy paper[a,b]
- Chlorotalonil[a,b]
- Citraconin anhydride
- Denatonium benzoate[a,b]?
- Di(2-ethyhexyl)phtalate[a]
- Dicyanidiamide
- Didecyl dimethyl ammonium chloride I[a]
- Diethylfumarate
- Diethyltoluamine[b]
- Dimethyl ammonium chloride[a]
- Didecyl dimethyl ammonium chloride
- Diclycidyl ether of bisphenol A[a,b]
- Formaldehyde resin [a,b]
- Fumaric acid
- Guanidinium salts[a]
- Methyl ethyl ketone[b]
- Monoamylamine[b]
- Naphta[a,c]
- Naphthylacetic acid
- Nitrile[a]
- Nylon[b]
- Oleylamide
- Phosphorus sesquisulfide
- Polypropylene[a]
- Potassium ferricyanide
- Sodium fluoride
- Sodium silicate
- Sodium sulfide
- Sulfur[c]
- Triphenyl phosphate[a]
- Trichloroethanol
- Uranium salts[a]
- Vinyl pyridine[a]
- Xylene[a]
- Zinc diethyldiothiocarbamate[a]
- Metals
  - Aluminium
  - Chromium[a,b]
  - Cobalt[a,b]
  - Cooper
  - Gold
  - Iridium[a,b]
  - Mercury[b]?
  - Nickel[a,b]
  - Palladium
  - Platinum salts[a,b]
  - Rhodium[a]
  - Ruthenium
  - Tin
  - Zinc

[a]Occupational.[b]Immunologic.[c]Nonimmunologic.

CHALLENGES AND FURTHER RESEARCH IN CONTACT URTICARIA SYNDROME

The knowledge of CUS still shows some challenges that need further research. Until now we assume new cases as exceptional findings adding each year new triggers to long lists of substances. General population-based epidemiologic studies are still missing. Proteins or low–molecular weight chemicals can be responsible for clinical manifestations, urticaria, or eczema, a consequence of different pathogenic mechanisms. Sometimes the same substance can induce both clinical patterns. This fact opens the door for new insights into new immune system pathways. Substances responsible for immediate contact skin reactions can be classified by molecular weight, mechanism of action, occupational relevance, or their common use in our daily life. It will be useful to replace in vivo tests by effective in vitro testing for diagnostic purposes. After symptoms control an appropriate etiologic diagnosis and the development of concrete preventive measures is required. The CUS is a worldwide health problem that needs a global approach.

RECOMMENDED TEXT BOOKS

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Chowdhury MMU, Maibach HI, eds. Latex Intolerance: Basic Science, Epidemiology and Clinical Management. Boca Raton, New York: CRC Press, 2005.

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15 Percutaneous penetration enhancers: An overview

Sailesh Konda, Haw-Yueh Thong, and Howard I. Maibach

INTRODUCTION

Skin is an optimal interface for systemic drug administration. Transdermal drug delivery (TDD) is the controlled release of drugs through intact or altered skin to obtain therapeutic levels systemically and to affect specified targets for the purpose of, for example, blood pressure control, pain management, and others. Dermal drug delivery (DDD) is similar to TDD except that the specified target is the skin itself (1). TDD has the advantages of bypassing gastrointestinal incompatibility and hepatic "first pass" effect; reduction of side effects due to the optimization of the blood concentration–time profile; predictable and extended duration of activity; patient-activated/patient-modulated delivery; elimination of multiple-dosing schedules, thus enhancing patient compliance; minimization of inter- and intrapatient variability; reversibility of drug delivery allowing the removal of drug source; and relatively large area of application comparing with the mucosal surfaces (1).

After four decades of extensive study, the success of this technology remains limited, with many problems waiting to be solved, one of which is the challenge of low skin permeability hindering the development of TDD for macromolecules. To overcome the skin barrier safely and reversibly, while enabling the penetration of macromolecules, is a fundamental problem in the field of TDD and DDD.

Several technologic advances have overcome skin barrier properties (2). Examples include physical means, such as iontophoresis, sonophoresis, microneedles; chemical means using penetration enhancers (PEs); and biochemical means, such as liposomal vesicles and enzyme inhibition.

This overview covers physical and biochemical means of penetration enhancement and focuses on the common chemical PEs. We discuss the classification and mechanisms of chemical PEs, their applications in TDD, and trends and development in penetration enhancement.

PHYSICAL PENETRATION ENHANCEMENT

Physical means of penetration enhancement mainly incorporate mechanisms to transiently circumvent the normal barrier function of stratum corneum (SC) and to allow the passage of macromolecules. Although the mechanisms are different, these methods share the common goal to disrupt SC structure to create "holes" big enough for molecules to permeate. The better-known technologies are iontophoresis and electroporation sonophoresis. Iontophoresis utilizes bipolar electrical fields to propel charged macromolecules across intact skin and into the underlying tissue. The active electrode has the same charge as the penetrant and effectively repels the ionic macromolecules and forces migration across the skin. Novel iontophoretic patch delivery systems have been developed for several macromolecules, including sumatriptan (3). Electroporation induces nanometer-sized transient pores in cell membranes by the application of short duration, high-intensity electric field pulses that generate transmembrane potentials of 0.5–1.0 V and last for 10 μs to 10 ms. This results in spherical deformations, a network-like structure, and multilamellar vesicles, which could be secondary to the heating effect of the pulse (4). Sonophoresis (phonophoresis) uses sonic waves or ultrasound to produce cavitation, which causes the formation of microchannels called lacunae in the corneocytes, enlargement of intercellular spaces, and perturbation of SC lipids. Macromolecules can penetrate the skin through these lacunae.

Microporation involves the creation of micro-sized micropores or microchannels in the skin, which can allow for the transport of macromolecules. These technologies include microneedle-enhanced delivery, thermal or radiofrequency ablation, and laser ablation. Microneedle-enhanced delivery uses arrays of tiny needle-like structures to create transport pathways of micron dimensions, and permits transport of macromolecules, possibly supramolecular complexes and microparticles. These systems have greatly enhanced (up to 100,000-fold) the penetration of macromolecules through skin (5), although offering painless drug delivery (6,7).

Laser microporation utilizes an Erbium:YAG laser to ablate the skin by conventional or fractional modalities. The efficacy of the fractional modality has been explored with macromolecules, such as 5-aminolevulinic acid, with minimal skin disruption (8). Fractional photothermolysis thermally ablates hundreds of microscopic columns of epidermal and dermal tissue in regularly spaced arrays separated by islands of untreated skin, which enable rapid migration and proliferation of keratinocytes to repair the damaged areas. These columns of tissue are known as microthermal treatment zones and can facilitate the passage of macromolecules. Similarly, thermal and radiofrequency microporation use arrays of filaments or microelectrodes to locally ablate the SC and create micropores, which lower the resistance for macromolecular diffusion.

Dermaportation utilizes electromagnetic pulses to enhance skin permeation and push target macromolecules away from the field. It utilizes a low voltage (3 V) and does not require direct physical contact with the skin to produce diffusion enhancement, which is

TABLE 15.1
Physical Methods of Penetration Enhancement

Method	Definition	Mechanism(s)	Examples of Drugs
Iontophoresis	The electrical driving of charged molecules into tissue by passing a small direct current through a drug-containing electrode in contact with skin	Electrical repulsion from the driving electrode drives charged molecules; flow of electric current enhances skin permeability; electroosmosis affects uncharged and large polar molecules	Calcitonin, transnail delivery of salicylic acid, lidocaine, dexamethasone, pilocarpine, iron, sumatriptan, transdermal delivery of peptides, proteins, and oligonucleotides
Electroporation	A method of reversibly permeabilizing lipid bilayers by the application of an electric pulse	Application of short (micro- to millisecond) electrical pulses of ~100–1000 V/cm creates transient aqueous pores in the lipid bilayers	Methotrexate, timolol, fentanyl, tetracaine, nalpuphine, cyclosporin-A
Sonoporation	Ultrasound mediated delivery of therapeutic agents into biological cells	Low-energy frequency disturbs the lipid packing in SC by cavitation; shock waves increase free volume in bimolecular leaflets, thus enhancing permeation	Insulin, cutaneous vaccination, transdermal heparin delivery, transdermal glucose monitoring, delivery of acetyl cholinesterase inhibitors for the treatment of Alzheimer's disease, treatment of bone diseases and Peyronie's disease, and dermal exposure assessment
Microneedle-enhanced delivery systems	A method using arrays of microscopic needles to open pores in SC, thus facilitating drug permeation	Bypasses the SC and delivers drugs directly to the skin capillaries; advantage of being too short to stimulate pain fibers	Oligonucleotide, insulin, protein vaccine, DNA vaccine, methyl nicotinate
Laser microporation	Laser ablation of skin using a conventional erbium:YAG or fractionated erbium:YAG laser	Conventional epidermal ablation removes SC barrier; fractionated epidermal ablation creates numerous microscopic zones of damage in the skin surrounded by islands of normal tissue	Lidocaine, 5-aminolevulinic acid, preclinical work in diabetes, pain, vaccine, and in vitro fertilization therapeutic areas
Thermal microporation	Array of metallic filaments attached to conventional patch are activated by a handheld applicator by a single pulse of electrical energy	Energy is converted to thermal energy which ablates the SC under each filament to create micropores	Interferon-α, hepatitis B antigen, parathyroid hormone
Radiofrequency microporation	High frequency AC current passed through a densely spaced array of microelectrodes on skin to create localized ablation	Creates RF-microchannels with a typical depth of <100 μm and covering <% of treated area	hPTH 1–34, human growth hormone, insulin, granisetron, diclofenac, plasmid DNA, nanoparticles
Dermaportation	Electromagnetic pulses enhance skin permeation and push target molecules away from the field	Influences molecular movement of drug molecules in epidermis and the structure of SC lipid bilayers	A-aminolevulinic acid, diclofenac, lidocaine

Abbreviations: hPTH, human parathyroid hormone; SC, stratum corneum.
Source: Adapted from Ref. 44.

particularly useful in drug delivery for wound management (9). The proposed mechanism is that the magnetic field influences both the molecular movement of macromolecules in the epidermis and the ordered structure of the SC lipid bilayers. Table 15.1 summarizes the commonly investigated technologies of physical penetration enhancement.

BIOCHEMICAL PENETRATION ENHANCEMENT

Biochemical means of penetration enhancement includes using prodrug molecules (10), chemical modification (11), enzyme inhibition (12), and the usage of vesicular systems or colloidal particles (13). Among these strategies, special formulation approaches based mainly on the usage of colloidal particles are most promising. Liposomes (phospholipids-based artificial vesicles) and niosomes (nonionic surfactant vesicles) are widely used to enhance drug delivery across the skin and have proved efficacious with drugs ranging from colchicine to minoxidil (14,15). In addition, proliposomes and proniosomes, which are converted into liposomes and niosomes upon simple hydration, are also used in TDD (16). Generally, these colloidal carriers are not expected to penetrate into viable skin. Most reports cite a localizing effect whereby the carriers accumulate in SC or other upper skin layers (5).

More recently, a new type of liposomes called transferosomes have been introduced (17,18). Transferosomes consist of phospholipids, cholesterol, and additional "edge activators"—surfactant molecules, such as sodium cholate. The inventors claim

that 200–300 nm-sized transferosomes are ultradeformable and squeeze through pores less than one-tenth of their diameter, and are thus able to penetrate intact skin. Penetration of these colloidal particles works best under in vivo conditions and requires a hydration gradient from the skin surface toward the viable tissues to encourage skin penetration under nonoccluded conditions.

In addition, ethosomes, which are liposomes high in ethanol content (up to 45%), penetrate skin and enhance compound delivery to deep skin strata or systemically (19). The mechanisms suggested are that ethanol induces vasodilation and increases the fluidity of SC lipid bilayers through lipid peroxidation, allowing the soft, malleable vesicles to penetrate through the disorganized lipid bilayers (20). Interestingly, both acute and chronic ethanol consumption has been shown to increase transdermal penetration of topically applied xenobiotics (21).

Emulsions and microemulsions are clear, stable, isotropic mixtures of oil, water, and surfactant, frequently found in combination with a cosurfactant. In contrast to emulsions, microemulsions that form upon simple mixing of the components, do not require high shear conditions, and can be prepared by an inexpensive process through autoemulsification. These biphasic systems (hydrophilic and hydrophobic) are convenient because they allow for the placement of compounds based on solubility and stability (22). Recently, positively charged emulsions, submicron emulsions, and microemulsions have been used as drug carriers based on their attraction to the negatively charged protein residues on the outer side of epithelial cell membranes (23).

In general, six potential mechanisms of action of these colloidal carriers were proposed (5):

1. Penetration of SC by a free drug process—drug releases from vesicle and then penetrates skin independently.
2. Penetration of SC by intact liposomes.
3. Enhancement due to release of lipids from carriers and interaction with SC lipids.
4. Improved drug uptake by skin.
5. Different enhancement efficiencies control drug input.
6. The role of protein requires elaboration.

CHEMICAL PENETRATION ENHANCERS

Substances that help promote drug diffusion through the SC and epidermis are referred to as PEs, accelerants, adjuvants, or sorption promoters (24). PEs have been extensively studied given their advantages, such as design flexibility with formulation chemistry and patch application over large areas. PEs improve drug transport by reducing the resistance of SC to drug permeation. Even water can function as a PE via soaking, occlusion, and high humidity, allowing for increased elasticity and permeability of the SC (25).To date, none of the existing chemical penetration enhancers (CPEs) has proved to be ideal. In particular, the efficacy of PEs toward the delivery of high–molecular weight drugs remains limited. Attempts to improve the enhancement by increasing the potency of enhancers inevitably leads to a compromise on safety issues. Achieving sufficient potency without irritancy has proved challenging.

CLASSIFICATION OF CPEs

The diverse physicochemical properties and variation in mechanisms of action of compounds investigated for their penetration enhancement effects make it difficult to set up a simple classification scheme for PEs. Hori and colleagues proposed a conceptual diagrammatic approach based on Fujita's data for the classification of PEs (26,27). In this approach, they determined organic and inorganic values for PEs, and the resultant plot of organic *versus* inorganic characteristics grouped PEs into distinct areas on the diagram—area I encloses enhancers, which are solvent, area II designates PEs for hydrophilic drugs, and area III contains PEs for lipophilic compounds. However, Lambert and colleagues grouped most PEs into three classes: solvents and hydrogen bond acceptors (e.g., dimethylsulfoxide, dimethylacetamide, and dimethylformamide), simple fatty acids and alcohols, and weak surfactants containing a moderately sized polar group (e.g., Azone®, 1-dodecylazacycloheptan-2-one), whereas Pfister and colleagues classified PEs as either polar or nonpolar (24,28). To date, there is no consensus as to which classification to adopt. Table 15.2 classifies commonly investigated PEs based on the chemical classes to which the compounds belong (29). Only representative compounds are listed to avoid an exhaustive list. Additional references can be found in "Dermatotoxicology," 7th edition or "Smith's textbook" (1st and 2nd editions) (2,30,31). Note that a perfect classification is yet to be developed and the key lies in a comprehensive understanding of the mechanisms and the physicochemical parameters of CPEs.

Mechanisms of CPEs

The mechanisms of action proposed for commonly seen CPEs are listed in Table 15.2. Basically, transdermal penetration of most drugs is a passive diffusion process (32). There are three major potential routes for penetration—appendageal (through sweat ducts or hair follicles with associated sebaceous glands), transcellular permeation through the SC, or intercellular permeation through the SC (5). The intact SC comprises the predominant route through which most molecules penetrate. On the other hand, despite its small available fractional area of 0.1%, the appendageal route, especially the follicular route, has recently received considerable attention and was found to be an important penetration pathway and a possible space for an intracutaneous reservoir (33,34). Liposomal formulations have shown to be useful delivery systems for follicular drug targeting (35), and transfollicular drug delivery seems promising for gene therapy and vaccination (36,37).

Kanikkannan and colleagues suggested three pathways for drug penetration through the skin: polar, nonpolar, and both (38). The mechanism of penetration through the polar pathway is to cause protein conformational change or solvent swelling, whereas the key to penetrate via the nonpolar pathway is to alter the rigidity of the lipid structure and liquefy the crystalline pathway. Some enhancers may act on both polar and nonpolar pathways by dissolving the skin lipids or denaturing skin proteins. However, Ogiso and Tanino proposed the following mechanisms for the enhancement effect: (*i*) an increase in the fluidity of the SC lipids and reduction in the diffusional resistance to permeants, (*ii*) the removal of intercellular lipids and dilation between adherent cornified cells, (*iii*) an increase in the thermodynamic activity of drugs in vehicles, and (*iv*) the exfoliation of SC cell membranes, the dissociation of adherent cornified cells, and elimination of the barrier function (39).

Ogiso and colleagues also proposed examples of PEs with different relative enhancement capabilities due to differences in the chemical structure and other parameters (40). In their study, the

TABLE 15.2
Chemical Penetration Enhancers

Category and Examples	Cosolvent/Vehicle	Mechanism	Examples of Drugs (53)	Comment
Sulfoxides				
DMSO		Increases lipid fluidity; promotes drug partitioning	DMSO: theophylline, salicylic acid, hydrocortisone, testosterone, scopolamine, antimycotics, fluocinolone acetonide, flufenamic acid	
DCMS		Protein-DCMS interactions, resulting in a change in protein conformation, creating aqueous channels	DCMS: methotrexate, naloxone, pyridostigmine bromide, hydrocortisone, progesterone	DCMS enhance polar drugs more effectively
Alkanones				
N-heptane, *N*-octane, *N*-nonane, *N*-decane, *N*-undecane, *N*-dodecade, *N*-tridecane, *N*-tetradecane, *N*-hexadecane		Extensive barrier alteration of SC	Propanol, diazepam	
Alcohols				
Alkanol: Ethanol (E), propanol, butanol, 2-butanol, pentanol, 2-pentanol, hexanol, octanol, nonanol, decanol, benzyl alcohol (BA)		Low molecular weight alkanols (C ≤ 6) may act as solubilizing agents; more hydrophobic alkanols may extract lipids from SC, leading to increased diffusion	E: tacrine, metrifonate, dichlorvos, ketolorac, nitroglycerin, tazifylline, betahistine, cyclosporin A	
Fatty alcohol: Caprylic, decyl; lauryl (LA), 2-lauryl, myristyl, cetyl, stearyl, oleyl, linoleyl, linolenyl alcohol			LA: buprenorphine	
Polyols				
PG, PEG, ethylene glycol, diethylene glycol, triethylene glycol, dipropylene glycol, glycerol (G), propanediol, butanediol, pentanediol, hexanetriol	43× Enhancement of diazepam and 86× enhancement of midazolam maleate seen in PG and 5% Azone in a PG:E:water (2:2:1) vehicle	PG may solvate α-keratin and occupy hydrogen bonding sites, reducing drug-tissue binding	PG: 5-fluorouracil, tacrine, ketorolac, isosorbide dinitrate, clonazepam, albuterol, verapamil, betahistine, estradiol, dihydroergotamine, methotrexate, steroids, midazolam maleate, diazepam PEG: terbutaline G: diazepam, terbutaline, 5-fluorouracil	Inclusion of 2% Azone or 5% oleic acid to PG produced a more bioactive formulation
Amides				
Urea, DMA, diethyltoluamide, DMF, dimethyloctamide, dimethyldecamide		Urea: hydration of SC, keratolytic, creating hydrophilic diffusion channels DMA/DMF: (low concentration): partition to keratin, (high concentration): increase lipid fluidity, disrupt lipid packaging	Urea: ketoprofen, 5-fluorouracil DMA/DMF: griseofulvin, betamethasone 17-benzoate, caffeine	Urea analogs in PG enhanced permeability of 5-fluorouracil 6x
Biodegradable cyclic urea: 1-alkyl-4-imidazolin-2-one			Indomethacin	Comparable to or better than Azone

Pyrrolidone derivatives: 1M2P, 2-pyrrolidone, 1-lauryl-2-pyrrolidone, 1-methyl-4-carboxy-2-pyrrolidone, 1-hexyl-4-carboxy-2-pyrrolidone, 1-lauryl-4-carboxy-2-pyrrolidone, 1-methyl-4-methoxycarbonyl-2-pyrrolidone, 1-hexyl-4-methoxycarbonyl-2- pyrrolidone, 1-lauryl-4-methoxycarbonyl-2-pyrrolidone, NMP, *N*-cyclohexylpyrrolidone, *N*-dimethylaminopropylpyrrolidone, *N*-cocoalkypyrrolidone, *N*-tallowalkylpyrrolidone Biodegradable pyrrolidone derivatives: Fatty acid esters of *N*-(2-hydroxyethyl)-2-pyrrolidone		Interact with both keratin in the SC and with lipids in the skin structure	1M2P: griseofulvin, theophylline, tetracycline, ibuprofen, betamethasone 17-benzoate NMP: prazosin	
Cyclic amides: 1-dodecylazacycloheptane-2-one (Azone), 1-geranylazacycloheptan-2-one, 1-farnesylazacycloheptan-2-one, 1-geranylgeranylazacycloheptan-2-one, 1-(3,7-dimethyloctyl)azacycloheptan-2-one, 1-(3,7,11-trimethyldodecyl) azacyclohaptan-2-one, 1-geranylazacyclohexane-2-one, 1-geran ylazacyclopentan-2,5-dione, 1-farnesylazacyclopentan-2-one Hexamethylenelauramide and its derivatives Diethanolamine, triethanolamine	Azone: enhancer effect can be increased by use of cosolvent such as PG	Azone: affects lipid structure of SC; increases partitioning; increases membrane fluidity	Azone: 5-fluorouracil, antibiotics, glucocorticoids, peptites, clonazepam, albuterol, estradiol, levonorgestrel, HIV protease inhibitor (LB-71148), betahistine, dihydroergotamine	Azone: significant accelerant effects at low concentration (1–5%), can be applied undiluted to skin without significant discomfort, effective for both hydrophilic and hydrophobic drugs
Fatty acids Linear: LIA, valeric, haptanoic, pelagonic, caproic, CA, LAA, myristic, stearic, OA, caprylic Branched: Isovaleric, neopentanoic, neoheptanoic, neononanoic, trimethyl hexanoic, neodecanoic, isostearic		Selective perturbation of the intercellular lipid bilayers OA: decreases phase transition temperatures of lipids, which increases their motional freedom or fluidity	Naloxone, mannitol, betamethasone 17-benzoate, hydrocortisone, acyclovir, nitroglycerin OA: galanthamine, estradiol, levonorgestrel CA: buprenorphine, albuterol LAA: buprenorphine, betahistine	Among stearic, oleic, and linoleic acids, maximum enhancement was observed with linoleic acid
Fatty acid esters Aliphatic: Isopropyl *n*-butyrate, isopropyl *n*-hexanoate, isopropyl *n*-decanoate, IPM, isopropyl palmitate, octyldodecyl myristate Alkyl: EA, butyl acetate, methyl acetate, methylvalerate, methylpropionate, diethyl sebacate, ethyl oleate		IPM: direct action on SC, permeating into liposome bilayers, increasing fluidity Aliphatic: increase diffusivity in the SC and the partition coefficient Alkyl: increase lipid fluidity (similar to DMSO)	IPM: galanthamine, ketorolac, chlorpheniramine, dexbrompheniramine, diphenhydramine, theophylline, pilocarpine, verapamil EA: levonorgestrel, 17β-estradiol, hydrocortisone, 5-fluorouracil, nifedipine	

Surfactants				
Anionic: Sodium laurate, sodium lauryl sulfate, sodium octyl sulfate		Alter the barrier function of SC, allowing removal of water-soluble agents that normally act as plasticizers		Greater damage and permeation enhancement with anionic surfactants than with nonionic surfactants
Cationic: Cetyltrimethylammonium bromide, tetradecyltrimethylammonium bromide, octyltrimethyl ammonium bromide, benzalkonium chloride, octadecyltrimethylammonium chloride, cetylpyridinium chloride, dodecyltrimethylammonium chloride, hexadecyltrimethylammonium chloride	Significant increases in the flux of lidocaine from saturated systems in PG-water mixtures	Adsorb at interfaces and interact with biological membranes, causing damage to skin		Cationic surfactants are more destructive to skin than anionic surfactants
Zwitterionic surfactants: Hexadecyl trimethyl ammoniopropane sulfonate, oleyl betaine, cocamidopropyl hydroxysultaine, cocamidopropyl betaine				
Nonionics: Polyxamer 231,182,184, polysorbate (20),60, brij (30),93,96,99, span (20,40),60,80,85, tween (20,40),60,80, myrj (45,51,52), miglyol 840	Polysorbate 20 and 60 increased lidocaine flux in the presence of PG	Emulsify sebum, enhancing the thermodynamic activity of coefficients of drugs	Tween 80: ketoprofen Polysorbate 20,60: lidocaine	
Bile salts: Sodium cholate, sodium salts of TC, glycolic, desoxycholic acids			TC: elcatonin and vit D_3, estradiol and vit D_3	
Lecithin				
Terpenes				
Hydrocarbons: *d*-limonene, α-pinene, β-carene		Increase diffusivity of drugs within SC due to disruption of intercellular lipid bilayer; open new polar pathways within and across the SC	5-Fluorouracil, aspirin, haloperidol, labetolol	Hydrocarbon terpenoids were least effective; oxides moderately effective; and the alcohols, ketones, and cyclic ethers most effective accelerants of 5-fluorouracil permeation
Alcohols: α-Terpineol, terpinen-4-ol, carvol				
Ketones: Carvone, pulegone, piperitone, menthone				
Oxides: Cyclohexene oxide, limonene oxide, α-pinene oxide, cyclopentene oxide, 1,8-cineole				
Oils: Ylang ylang, anise, chenopodium, eucalyptus, basil, menthol				
Organic acids Salicylic acid and salicylates (including their methyl, ethyl, and propyl glycol derivatives), citric and succinic acid				

Cyclodextrins			
HPβCD, DIMEB	Higher penetration of liarozole in DIMEB with PG/oleic acid compared to HPβCD	Form inclusion complexes with lipophilic drugs and increase their solubility in aqueous solutions	Liarozole
Proprietary chemical enhancers			
Alkyl-2-(*N*,*N*-distributed amino)-alkanoate ester (NexAct®), 2-(*n*-nonyl)-1 ,3-dioxolane (SEPA®)			Ibuprofen, ketoprofen, alprostadil, testosterone

Source: Adapted from Ref. 29.

Abbreviations: BA, benzyl alcohol; CA, capric acid; DCMS, Decylmethylsulfoxide; DIMEB, 2,6-dimethyl-β-cyclodextrin; DMA, dimethylacetamide; DMF, dimethylformamide; DMSO, dimethylsulfoxide; E, ethanol; EA, ethyl acetate; G, glycerol; HPβCD, 2-hydroxypropyl-βcyclodextrin; IPM, isopropyl n-decanoate, isopropyl myristate; LA, lauryl; LAA, lauric acid; LIA, linoleic; NMP, N-methyl-pyrrolidone; OA, oleic acid; PEG, polyethylene glycol; PG, propylene glycol; SC, stratum corneum; TC, sodium salts of taurocholic; 1M2P, 1-methyl-2-pyrrolidone.

TABLE 15.3
Examples of Penetration Enhancers with Different Relative Enhancement Capabilities

Mechanisms	Comparison
Extraction of intercellular lipids and dilations between cornified cells, permitting percutaneous passage of polar substances	1-Dodecylazacycloheptane-2-one (Azone) > *n*-octanol > *d*-limonene > oleic acid > cineol
Increase in partitioning into skin	1-Dodecylazacycloheptane-2-one > *n*-octanol > cineol > *d*-limonen > oleic acid > isopropyl myristate > monooleate
Increase in the fluidity of stratum corneum lipids and reduction in diffusional resistance	1-Dodecylazacycloheptane-2-one > isopropyl mysirate > monoolein > oleic acid > cineol, sodium oleate
Increase in thermodynamic activity in vehicles	*n*-Octanol > sodiumoleate > *d*-limonen > monoolein > cineol > oleyl oleate > isopropyl myristate

relative ability to enhance transdermal penetration of indomethacin into hairless rat skin was studied. The results are summarized in Table 15.3 (41).

Furthermore, Kanikkannan and colleagues proposed that based on the chemical structure of PEs (such as chain length, polarity, level of unsaturation, and presence of specific chemical groups, such as ketones), the interaction between the SC and PEs may vary, contributing to the different mechanisms in penetration enhancement (38). A comprehensive understanding of the mechanisms of action and a judicious selection of a CPE would be helpful in the successful development of TDD and DDD products.

U.S. FOOD AND DRUG ADMINISTRATION–APPROVED TDD

There has been an increased focus on the potential of transdermal drug delivery as evident from the increase in the number of patents as well as scientific publications on TDD systems. Many drugs have been evaluated for TDD in prototype patches, either in vitro permeation studies using mouse, rat, or human skin, or have reached varying stages of clinical testing. Examples are listed in Table 15.2. Despite a wide array of TDD systems undergoing research and development, only a small percentage of drugs successfully reaches the market due to three limitations: difficulty of penetration through human skin, skin irritation and allergenicity, and clinical need. In addition, it is generally accepted that the best drug candidates for passive adhesive transdermal patches must be nonionic, low molecular weight (less than 500 Da), have adequate solubility in oil and water (log *P* in the range 1–3), a low melting point (less than 200°C), and are potent (dose is less than 50 mg/day and ideally less than 10 mg/day) (42,43). Given these operating parameters, the number of drug candidates that fit these criteria may seem low. Nevertheless, we may overcome such constraints with the development of novel technologies.

Since the introduction of a TDD for scopolamine in 1981, several new products have been introduced. Transdermal patches currently on the market are limited to the delivery of small, potent, and lipophilic drug molecules, such as clonidine, estradiol, ethinyl estradiol, norethindrone acetate, testosterone, fentanyl, lidocaine, prilocaine, granisetron, methylphenidate, rivastigmine, rotigotine, selegiline, nicotine, nitroglycerin, oxybutynin, scopolamine, and buprenorphine. The U.S. TDD market approached $1.2 billion in 2001, $6.7 billion in 2006, and nearly $7.9 billion in 2010, signifying the increasing acceptance of TDD as a preferred method of administration for some macromolecules. Barry reported that 40% of drug delivery candidate products that were under clinical evaluation and 30% of those in preclinical development in the United States were TDD or DDD systems (5).

Examples of U.S. Food and Drug Administration–approved transdermal patches and their applications are listed in Table 15.4. Despite a plethora of candidate CPEs to choose from, all currently available TDD products adopt skin occlusion as the primary mechanism for penetration enhancement, perhaps due to its simplicity and convenience, and the following effects on SC (44–46): an increase in SC hydration and reservoir effect in penetration rates of the drug due to hydration, an increase in skin temperature from 32 to 37°C, and the prevention of accidental wiping or evaporation (volatile compound) of the applied compound.

FUTURE TRENDS

The protective function of human SC imposes physicochemical limitations to the type of molecules that can traverse the barrier. As a result, commercially available products based on TDD or DDD have been limited. Various strategies have emerged over the last decade to optimize delivery. Approaches such as the optimization of formulation or drug-carrying vehicle to increase skin permeability do not greatly improve the permeation of macromolecules.

Sufficient data on chemical enhancers is available, so that the "Smith's textbook" (1st and 2nd editions) provides extensive quantitative data (2,31). Note that of the several dozen proposed enhancers suggested over four decades, a few new chemical entities have received wide scale usage. Skin irritation by PEs may limit the use of these compounds in TDD. In general, the potency of PEs in causing skin irritation is proportional to their ability to cause fluidization of the lipid bilayer (47). Potent PEs cannot limit their activity to the superficial SC and eventually diffuse into viable epidermis to interact with keratinocytes and cause cytotoxicity. Attempts have been made to synthesize PEs that safely achieve therapeutic transport enhancement, such as 1-dodecylazacycloheptane-2-one (Azone) and 2-(n-nonyl)-1,3-dioxolane (SEPA®). Additionally, safer biodegradable enhancers can elicit an effect and then be broken down to inert compound and fatty acids in the subcorneal layers, where hydrolytic enzyme activity is present.

On the contrary, physical or mechanical methods of enhancing delivery have been more promising. Improved delivery has been shown for drugs of differing lipophilicity and molecular weight, including proteins, peptides, and oligonucleotides, using electrical methods (iontophoresis, electroporation), electromagnetic (dermaportation), mechanical (abrasion, ablation, perforation), and other energy-related techniques, such as ultrasound and needleless injection (48).

Another strategy for penetration enhancement is to exploit the synergistic effects offered by combined techniques. Karande and colleagues reported the discovery of synergistic combinations of PEs, which allow permeation of 10 kDa macromolecules with minimal skin irritation using a high-throughput screening method (49). Kogan and Garti also showed that the combination of several

TABLE 15.4
Examples of U.S. Food and Drug Administration–Approved Transdermal Patches, their Applications, and the Mechanisms/Compounds used for Penetration Enhancement

Drug	Application(s)	Example(s) of Commercially Available Product(s)	Penetration Enhancement Effect and Penetration Enhancers
Scopolamine	Motion sickness	Transderm Scop	Occlusive effect
Fentanyl	Moderate to severe chronic pain	Duragesic	Occlusive effect
Lidocaine	Anesthesia	Lidoderm	Occlusive effect, urea, propylene glycol
Prilocaine	Anesthesia	EMLA anesthetic disc	Occlusive effect, polyoxyethylene fatty acid esters
Testosterone	Hormone replacement therapy	Androderm	Occlusive effect, glycerol monooleate
Estradiol/norethindrone acetate	Hormone replacement therapy	Combipatch	Occlusive effect, silicone, oleic acid, dipropylene glycol
Estradiol	Symptomatic relief of postmenopausal symptoms and prevention of osteoporosis	Alora, climera, esclim, vivelle, vivelle-dot	Occlusive effect Climera: fatty acid esters Vivelle: 1,3-butylene glycerol, oleic acid, lecithin, propylene glycol, dipropylene glycol Vivelle-dot: oleyl alcohol, dipropylene glycol
Norelgestromin/ethinyl estradiol	Contraception	Ortho evra	Occlusive effect, lauryl lactate
Nitroglycerin	Angina pectoris	Nitro-Dur, nitrodisc, transderm-Nitro	Occlusive effect, fatty acid esters
Clonidine	Hypertension	Catapres-TTS	Occlusive effect
Nicotine	Smoking cessation	Nicoderm CQ	Occlusive effect
Methylphenidate	Attention-deficit hyperactive disorder	Daytrana	Occlusive effect
Selegiline	Parkinson's disease, depression, senile dementia	Emsam	Occlusive effect
Oxybutynin	Urge/urinary incontinence	Oxytrol	Occlusive effect
Granisetron	Nausea/emesis resulting from chemotherapy	Sancuso	Occlusive effect
Rivastigmine	Alzheimer's disease, Parkinson's disease	Exelon	Occlusive effect
Rotigotine	Parkinson's disease, restless legs syndrome	Neupro	Occlusive effect
Buprenorphine	Moderate to severe chronic pain	Norspan	Occlusive effect, oleyl oleate

enhancement techniques led to synergistic drug penetration and a decrease in skin toxicity (50). In essence, the possibilities seem endless in the field of TDD and DDD.

CONCLUSION

TDD would avoid problems associated with the oral route, as well as the inconvenience and pain associated with needle delivery; and has thus competed with oral and injection therapy for the accolade of the innovative research area for drug delivery. Yet there remains a paucity of candidates for TDD or DDD to be marketed. The reasons are twofold: (*i*) most candidate drug molecules have low permeation rates through the skin to ever reach a clinically satisfactory plasma level; (*ii*) risk of skin irritation and allergic contact dermatitis may be increased by skin occlusion (45,46) or the application of potent PEs (49). The ideal characteristics of PEs include the following (24):

- Be both pharmacologically and chemically inert
- Be chemically stable
- Have a high degree of potency with specific activity, rapid onset, predictable duration of activity, and reversible effects on skin properties (51)
- Show chemical and physical compatibility with formulation and system components
- Be nonirritant, nonallergenic, nonphototoxic, and noncomedogenic
- Be odorless, tasteless, colorless, cosmetically acceptable, and inexpensive
- Be readily formulated into dermatologic preparations, transdermal patches, and skin adhesives
- Have a solubility parameter approximating that of skin (i.e., 10.5 $cal^{1/2}/cm^{3/2}$) (52)

Future studies on the mechanisms of penetration enhancement, the metabolic processes of chemicals within the skin, skin toxicity, as well as the development of novel technologies will improve our knowledge on penetration enhancement. While the current TDD and DDD technologies still offer significant potential for growth, next-generation technologies will enable a much broader application of TDD to the biopharmaceutic industry.

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16 Chemical warfare agents

Robert P. Chilcott

INTRODUCTION

Human skin has evolved to impede the ingress of potentially toxic materials from the environment. However, the skin is not completely impermeable and many xenobiotics are able to penetrate the barrier layer (stratum corneum) to some extent. A number of chemicals have been specifically developed to exploit dermal absorption as a route of entry to deliberately induce local or systemic toxic effects. Collectively, these are known as chemical warfare (CW) agents. In contrast to other hazardous substances, such as toxic industrial chemicals (TICs), CW agents have little or no legitimate use; their sole purpose is to inflict harm. The percutaneous toxicity of CW agents is related to the rate and extent to which they undergo dermal absorption which, in turn, is dependent on a range of known factors, such as environmental exposure conditions and physicochemical properties of the agent. Therefore, the primary aim of this chapter is to examine the influence of these factors on the toxicity of CW agents.

RELEVANT SUBSTANCES

CW agents encompass a variety of substances, the possession and use of which are controlled under the Chemical Weapons Convention (1). Of these, a large proportion of high priority ("Schedule 1") materials are considered to be hazardous via dermal exposure (Table 16.1), with the most extensively studied examples of this genre being sulfur mustard (HD) and VX.

Sulfur mustard (bis(2-chloroethyl)sulfide) is an extremely potent blistering (vesicating) agent and can induce skin lesions following absorption of about 5–20 μg/cm^2 of liquid or vapor (2–4). Sulfur mustard is generally considered to be an incapacitating agent; the resulting skin lesions can be extensive, painful, and slower to heal than comparable (thermal) burns (5). The mechanism(s) through which sulfur mustard causes such extensive cutaneous damage is still unknown, although many hypotheses have been proposed (6–8).

Nerve agents such as VX exert their effect through inhibition of an enzyme [acetylcholinesterase (AChE)] that is critical for controlling synaptic transmission at cholinergic nerve endings (5). Inhibition of AChE may lead to overstimulation of smooth and skeletal muscles, resulting in death by respiratory paralysis. In contrast to sulfur mustard, dermal exposure to a single (5 mg) droplet of VX (O-ethyl S-(2-diisopropylaminoethyl) methyl phosphonothiolate) may be fatal if untreated (9).

FACTORS INFLUENCING PERCUTANEOUS ABSORPTION AND TOXICITY OF CW AGENTS

Factors that affect the dermal penetration of chemicals are dealt with in comprehensive detail in other chapters of this book. The purpose of this section is to describe examples of relevance to the dermal absorption of CW agents.

At a fundamental level, it is the dose of a chemical that dictates its toxicity. This was first recognized by Paracelsus (*aka* Theophrast von Hohenheim; *circa* 1493–1541) whose phrase "sola dosis facit venenum" (loosely translated as "the dose makes the poison") is a central paradigm of modern toxicology (10). This principle applies equally to the percutaneous toxicity of CW agents: factors which affect the rate and extent of skin absorption are necessarily factors that influence the percutaneous toxicity of CW agents.

Molecular Weight

It is interesting to note the molecular weight of substances listed in Table 16.1. The architecture of the stratum corneum is such that molecules in excess of 500 Da are generally unable to diffuse through this layer (Fig. 16.1). This is commonly referred to as the "rule of 500" (11). Thus, larger compounds, such as botulinum toxin, are essentially harmless in terms of percutaneous toxicity despite being lethal at very low doses when administered through respiratory, oral, or parenteral routes.

Volatility and Occlusion

At an empirical level, volatility exerts a time-dependent effect on dermal absorption: the higher the volatility, the less contact time is available for a chemical liquid to partition into the stratum corneum and subsequently penetrate. This does not take into account the ability of many chemicals to penetrate the skin in vapor or gas form, a classic example of which is sulfur mustard, which can elicit skin lesions following exposure to either the liquid or vapor (12).

The effect of volatility on the relative toxicity of different nerve agents is illustrated in Figure 16.2: in this example, the relative toxicity of each agent is calculated by dividing the percutaneous toxicity value (LD_{50}) of each chemical by the corresponding intravenous toxicity value. It can be seen that for the least volatile agent (VX), the ratio of percutaneous to intravenous toxicity approaches unity. That is, VX is nearly as toxic via percutaneous absorption as when delivered intravenously. In contrast, the most volatile agent (GB) is nearly three orders of magnitude less toxic when administered via the percutaneous route.

Occlusion refers to the situation where a permeable or semipermeable material covers the skin exposure site. In practical terms, this could potentially be normal clothing or protective garments (in the case of an emergency responder). The effects of occlusion are primarily related to a compound's volatility, although occlusion may also affect the hydration status and thus permeability of the stratum corneum (13).

TABLE 16.1
Chemical Warfare Agents Which Are Active Via the Percutaneous Route

Primary Effect	SMD	Chemical Name	Synonyms	MW
Local (cutaneous)	CX	Dichloroformoxime	Phosgene Oxime, nettle gas	114
	HD	bis-(2-chloroethyl)sulfide	Sulfur mustard, mustard gas, Yprite, Yellow Cross, Kampstoff "Lost"	160
	HL	—	Sulfur mustard/ Lewisite mixture	N/A
	HN-1	2,2′-Dichlorotriethylamine	Nitrogen mustard	170
	L	Dichloro(2-chlorovinyl)arsine	Lewisite	207
	MD	Methyldichloroarsine	Methyl-dick	160
	Q	1,2-bis (2-chloroethylthio) ethane	Sesqui-mustard	219
	T	bis[2-(2-chloroethylthio)ethyl] ether	—	263
	T2	—	T2 Mycotoxin, "Yellow rain"	467
Systemic	GA	Ethyl *N*,*N*-dimethylphosphoroamidocyanidate	Tabun	162
	GB	Isopropylmethylphosphonofluoridate	Sarin	140
	GD	Pinacolylmethylphosphonofluoridate	Soman	182
	GF	Cyclohexylmethylphosphonofluoridate	Cyclosarin, CMPF	180
	VX	O-ethyl S-(2-diisopropylaminoethyl) methyl-phosphonothiolate	-	267

Abbreviations: MW, molecular weight; SMD, standard military designation.

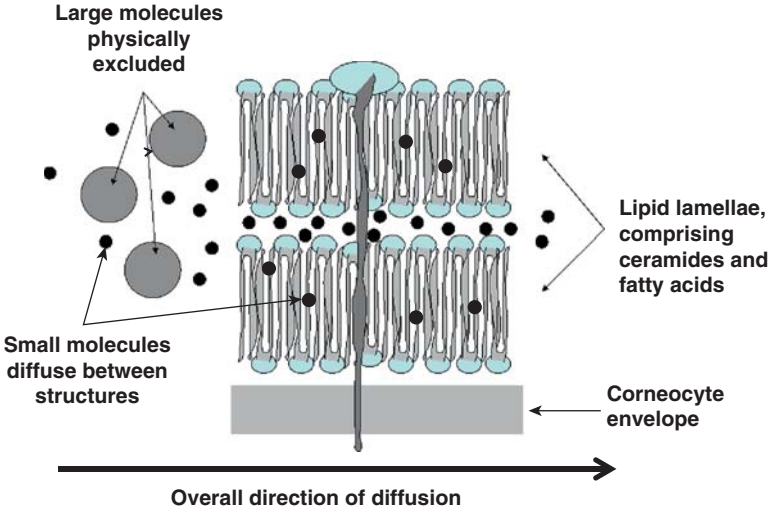

FIGURE 16.1 Schematic representation of stratum corneum, illustrating exclusion of heavier molecules from within lipid lamellae between corneocytes.

At one extreme, total occlusion may prevent evaporative loss of a volatile chemical from the skin surface and thus increase dermal absorption. Conversely, an unoccluded site will allow unimpeded vapor loss and so reduce dermal absorption. In the case of sulfur mustard, there is an 11-fold difference in the maximum rate of penetration and a 5-fold difference in the total absorbed dose under occluded and unoccluded conditions (Fig. 16.3). The latter is commensurate with an 80% evaporative loss of liquid under unoccluded conditions. Incidentally, this measurement of evaporative loss is identical between in vitro (14) and in vivo (4) experimental systems.

Anatomic Variation

It has long been acknowledged that skin permeability varies according to anatomic location (15,16). There is a common misconception that this is due to regional variations in skin thickness (17). The rate at which CW agents penetrate different areas of human skin can vary considerably (Fig. 16.4). In the case of the nerve agent VX, there is a 300-fold difference in the rate of percutaneous absorption between scrotal skin (most permeable) and the palm of the hand (18). An understanding of regional variation in skin absorption is necessary for predicting the effects of whole body exposures to CW agents, such as VX (19).

Skin Temperature

The rate at which a molecule undergoes percutaneous absorption is a function of its ability to diffuse within the stratum corneum (*D*), the applied concentration (*C*; or more accurately, thermodynamic activity), ability to partition into the skin (Km) and the thickness (*h*) of the stratum corneum (20). The interplay between these parameters forms the basis of Fick's law of diffusion (Equation 16.1).

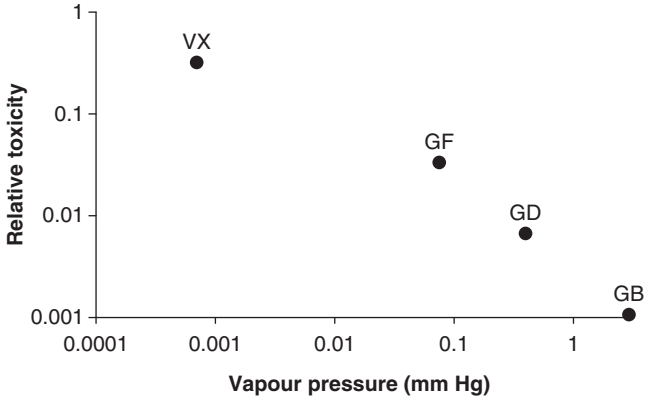

FIGURE 16.2 Relative toxicity of the nerve agents VX, GF (cyclosarin), GD (soman), and GB (sarin) as a function of volatility (expressed as vapor pressure). Relative toxicity expressed as the ratio of lethal dose (LD_{50}) between percutaneous and intravenous routes; a relative toxicity value of 1 indicates that both routes of administration result in an equitoxic response. *Source*: From Refs. 5,9.

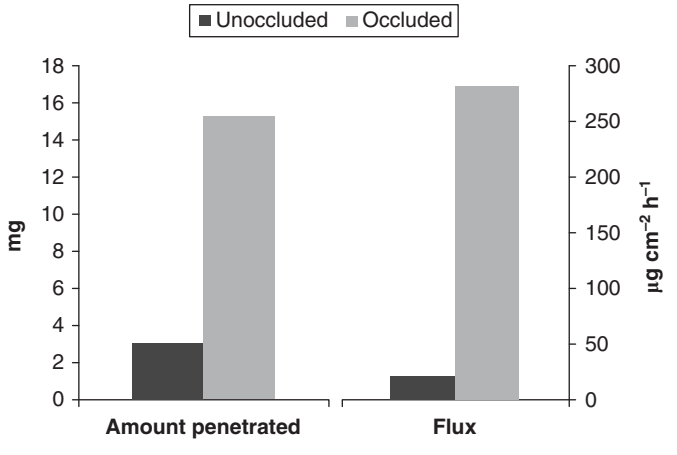

FIGURE 16.3 Dermal absorption kinetics (expressed as amount penetrated and maximum penetration rate; flux) of sulfur mustard through human skin in vitro under occluded and unoccluded conditions. *Source*: From Ref. 14.

$$J = \frac{D.C.Km}{h} \qquad (16.1)$$

The diffusivity coefficient is, in turn, governed by temperature according to Equation 2, where D_0 is the diffusivity coefficient (at infinite temperature), Ea is the activation energy, R is the molar gas constant, and T is the absolute temperature.

$$D = D_0 e^{-\frac{Ea}{RT}} \qquad (16.2)$$

A practical interpretation of these equations is that an increase of 10°C in skin temperature will approximately double the rate of dermal absorption (Fig. 16.5, dotted line). In the case of the CW agent sulfur mustard, there is some deviation from this ideal situation (Fig. 16.5, solid line), with a larger than expected increase in skin absorption associated with skin surface temperatures between 25 and 40°C (possibly due to experimental artifacts related to the use of occlusive test conditions).

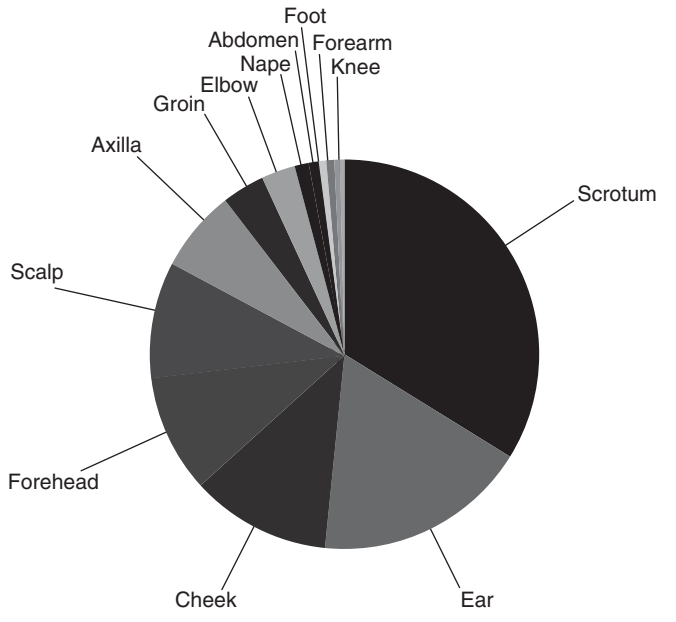

FIGURE 16.4 Relative permeability of human skin at different anatomic sites to the nerve agent VX (18). The permeability of each site was calculated from the rate of inhibition of blood cholinesterase acquired from human volunteers following topical application of VX. Values expressed relative to the least permeable site (palm of hand).

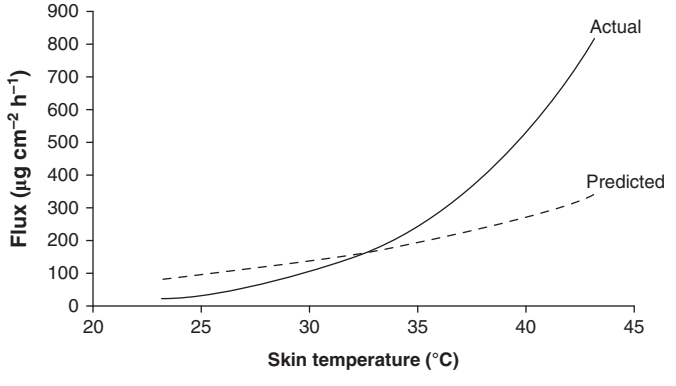

FIGURE 16.5 Effect of skin temperature on the percutaneous absorption of sulfur mustard. Penetration rates (flux) were calculated from the loss of radioactivity from the skin surface following 1 hour's exposure to a liquid droplet of radiolabeled sulfur mustard when applied under occlusive conditions to abdominal skin of human volunteers. *Source*: From Ref. 4.

Environmental Temperature

CW agents have been used under a variety of conditions, ranging from the cold, damp European winters of World War I to the hot, dry desert environment of the Persian Gulf (21). In the case of volatile CW agents, such as sarin, elevated temperatures will reduce the persistency and thus contact hazard associated with a liquid-contaminated environment. In contrast, less volatile CW agents, such as VX, may remain present even at relatively high temperatures and so continue to present a dermal hazard through accidental skin contact with contaminated surfaces. Environmental temperature is known to affect percutaneous absorption (22,23). Indeed, this has been demonstrated with the nerve agent VX (Fig. 16.6): a 5-fold difference was measured in the extent of dermal absorption between individuals placed in a climatic chamber at −10 or + 40°C (24).

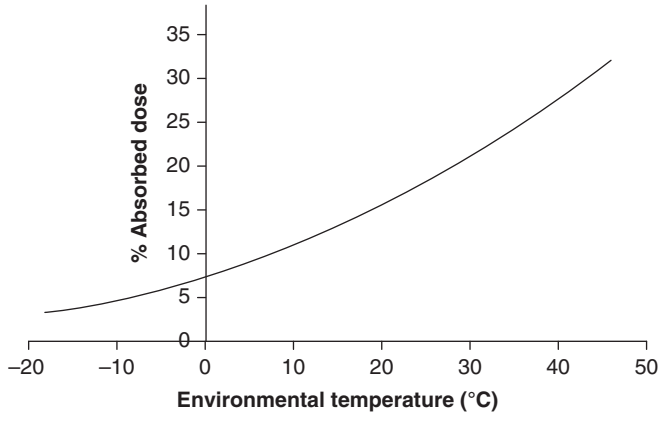

FIGURE 16.6 Effect of environmental temperature on dermal absorption of VX in human volunteers. Rate of absorption (expressed as a percentage of the applied dose penetrated; "% absorbed dose") was calculated 3 hours after the application of a liquid droplet to the face (cheek) of each individual *Source*: From Ref. 24.

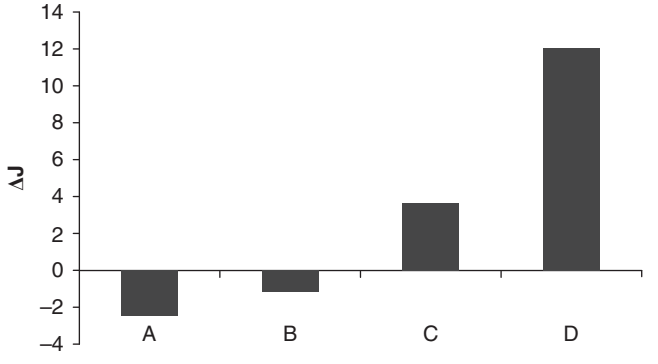

FIGURE 16.7 Effect of skin surface water on the dermal absorption of four chemical warfare agents (labeled A–D). The *y*-axis parameter (ΔJ) is the ratio of penetration rates measured through wet and dry skin. A negative ΔJ value indicates that the presence of water on the skin surface reduces dermal absorption, whereas a positive ΔJ value indicates an increase (unpublished data; see section on "Acknowledgments").

Skin Surface Conditions

In addition to the well-studied factors described above, percutaneous penetration is also subject to weather effects that directly or indirectly affect conditions on the skin surface, such as wetting (caused by rain) and sweating (elevated temperature). The toxicity of some CW agents may be mitigated by exposure to water. For example, Lewisite (a potent vesicant and systemic poison) is subject to hydrolysis and so may be less toxic via dermal exposure under wet conditions (25). However, the presence of water on the skin surface can also have a direct effect on percutaneous absorption, which may be difficult to predict. For example, rain water can reduce the concentration of a chemical on the skin surface and, in accordance with Fick's law of diffusion, lead to a decrease in dermal absorption (Fig. 16.7; chemicals A and B). Conversely, the presence of water may reduce the evaporative loss of a volatile chemical and/or act as a vehicle to enhance dermal absorption (Fig. 16.7; chemicals C and D). The latter effect has been demonstrated experimentally on human volunteers using sulfur mustard vapor, where prewetting of the skin resulted in more severe burns (26).

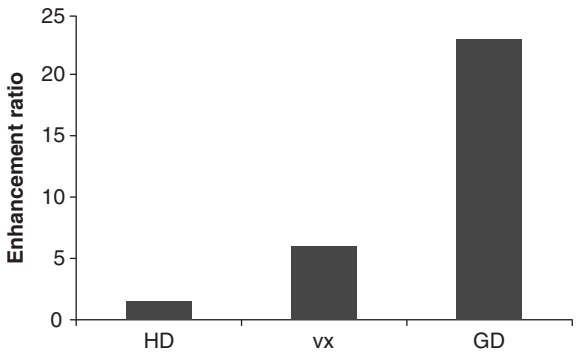

FIGURE 16.8 Effect of physical damage (expressed as enhancement ratio) on the in vitro dermal absorption of the chemical warfare agents sulfur mustard (HD), VX, and soman (GD). Enhancement ratio calculated as the difference in the total amount of chemical penetrating damaged or undamaged skin 24 hours postexposure (unpublished data; see section on "Acknowledgments").

A further factor to consider is the structural integrity of exposed skin sites: damage to the stratum corneum caused by abrasions or tape stripping will generally enhance the percutaneous absorption of xenobiotics, with the effect being more prominent with hydrophilic (water soluble) compounds (27,28). In a recent (unpublished) study, damage induced by the physical removal of epidermal tissue resulted in a 1.5- to 23-fold increase in the dermal absorption of CW agents (Fig. 16.8).

Other Considerations Relating to Dermal Absorption

Finally, there are two additional factors relating to the dermal absorption of CW agents, which have practical implications for the clinical management of exposed individuals: dermal absorption kinetics and the formation of reservoirs within the stratum corneum.

The dermal absorption kinetics of a contaminant may potentially affect the onset and duration of systemic exposure (29). For example, a CW agent that undergoes relatively slow dermal absorption may "outlive" standard antidotes, which have a shorter plasma half-life (Fig. 16.9). Therefore, it is important to understand the dermal absorption kinetics of skin contaminants to plan the optimum treatment strategies.

Topical exposure to chemical contaminants may result in the accumulation of material within the stratum corneum to form a reservoir of material that may potentially result in prolonged systemic absorption (30). Sulfur mustard, VX, and T2 mycotoxin are thought to form dermal reservoirs (31–33), removal of which may provide some clinical benefit (34). However, a dermal reservoir also provides a potential off-gassing hazard and so caution should be exercised when treating skin contaminated with CW agents (14,35,36).

SUMMARY

Factors that affect dermal absorption directly affect the percutaneous toxicity of CW agents. Those that exert the greatest influence include molecular weight, prevailing weather conditions, anatomic variation in permeability, and the volatility of the penetrant, the latter being largely influenced by the occlusive state of the skin exposure site. An understanding of the mechanisms that underpin the percutaneous absorption of CW agents is fundamental to the future development of effective medical countermeasures to reduce or eliminate the health effects of dermal exposure to such toxic materials.

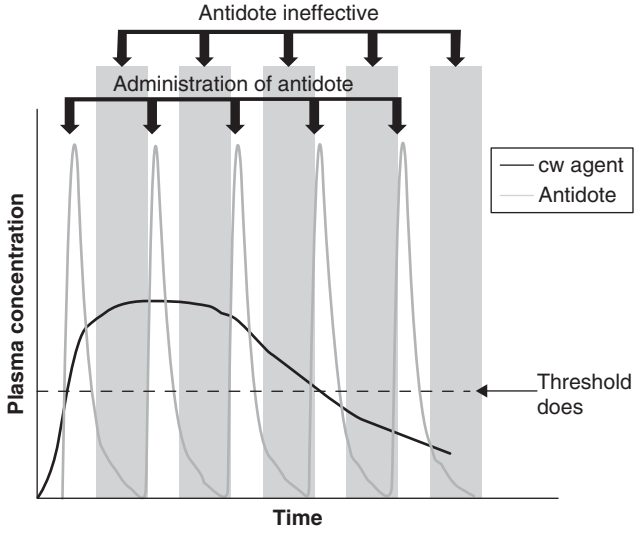

FIGURE 16.9 Hypothetical representation of plasma concentrations of a CW agent (following dermal exposure) and antidote (administered via intramuscular injection). Dotted line indicates threshold dose above which the CW agent produces overt toxic response or at which dose the antidote is effective. While the antidote may be initially effective, the plasma half-life is such that the dose falls below the minimum therapeutic level and so becomes ineffective. At this point, clinical signs of intoxication may recur. *Abbreviation*: CW, chemical warfare.

ACKNOWLEDGMENTS

The unpublished data referred to in this chapter was produced by Chris Dalton, Charlotte Hall, Rhys Jones, Joanne Larner, Helen Lydon, and Hazem Matar as part of their PhD studies with the financial support of the European Union Executive Agency for Health and Consumer, the Department of Health (England, UK), the UK Home Office, and the US Defence Threat Reduction Agency.

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17 Allergic contact dermatitis from ophthalmics

Andreas J. Brandstetter and Howard I. Maibach

INTRODUCTION

Allergic contact dermatitis (ACD) is a type IV hypersensitivity reaction. Langerhans cells internalize the allergen and transport it through the lymphatic system to the lymph nodes where they present it to T lymphocytes and activate them. These activated T lymphocytes induce the inflammatory reaction in the dermis. As for many other medications, there are cases of ACD from almost every group of ophthalmic drug. Here we want to give an overview of the reported cases and want to clarify, if there is an appropriate test to identify an allergic reaction caused by ophthalmics.

METHODS

We conducted a PubMed search for "allergic contact dermatitis" and "ophthalmic drugs" from January 2006 onward. The results from the previous reviews (1–4) have been merged in one table, which we updated with the new identified allergens.

RESULTS

Five new allergens have been reported: azithromycin, ketorolac, olapatadine, chlorobutanol, and *Myroxylon pereirae* (Peru Balsam).

COMMENT

Annotable to the olopatadine cases is that it was only retrospective, no patch test was done and the eyedrops contained benzalkonium chloride as preservative, which is a putative allergen (Table 17.1). There was no concentration and no vehicle given for *Myroxylon pereirae*.

DISCUSSION

Different kinds of trays and procedures make it problematic to compare and verify the case reports. The patch test is a step in diagnosing an allergy, although it has some limitations. The anatomic and physiologic properties of the eyelid are different from those of the back, where patch tests normally are performed. Scratching and tape stripping the skin would make it more comparable as it may lead to a higher percutaneous absorption (5). Therefore, after a negative result in patch testing, it should be repeated with the stripping and scratching technique. In addition many manufacturers have failed to provide drug samples for patch testing. As it is impossible to do a correct scientific analysis without the drug itself, presumably few cases are reported. All these factors follow frequent diagnostic errors and nonoptimal patient management.

Relating to this, Jappe et al. showed that only 16% of the patients who have been referred to their clinic with the suspicion of an ACD actually had ACD. The negative predictive value was 90% (6). As mentioned earlier, an efficient standardized international test, widely applied, would lead to more scientific usable data, which could be evaluated and, with that to an improved understanding of ACD, improved diagnostic and finally improved patient management.

Provocative use tests (repeat open application tests) to the eye remain a useful option when patch testing fails to disclose the allergy (6).

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TABLE 17.1
Drugs found to have an allergenic potential

Allergen (group)	PT Concentration (%)	Vehicle	PT-positive Cases	PT-negative Cases	References
Carbonic anhydrase inhibitors					
Dorzolamide	05/10	aq	1 cd	0/5	(7)
	10/15	pet	1 cd	0/5	(7)
	Eyedrops 2%	As is	1 cd	0/16	(8)
	0.0001/0.001/0/0.01/2	aq	1 cd	0/16	(8)
	40	ND	1 cd	0/10	(9)
	0.05/1	pet	1 cd	ND	(10)
	5	aq/pet	2 cd	0/10	(11)
Antioxidants					
Pirenoxone	Eyedrops (Catalin K)	As is	1 cd	ND	(12)
	0.005/1	aq	1 cd	ND	(12)
Sodium bisulfite	Eyedrops (Tathion)	As is	1 cd	0/10	(13)
	1/0.2	aq	1 cd		
Prostaglandins					
Latanoprost (PG)	Eyedrops (Xalatan)	As is	1 cd	ND	(14)
	1/0.2	aq	1 cd	ND	(14)
Corticosteroid					
Prednisolone	0.5	aq	1	ND	(15)
Antibiotics					
Vancomycin	0.005/5	aq	1 cd	ND	(16)
Sodium colistimethate	Eyedrops (Colimy C)	As is	1 cd	0/3	(17)
	1	aq/pet	1 cd	0/3	(17)
Tobramycin sulfate	5/25	pet	1	0/20	(18)
Bacitracin	20	ND	2 cc	0/24	(19)
Chloramphenicol	10	pet	1	0/3	(20)
	1.5	pet	3 cd	0/20	(21)
Gentamicin sulfate	20	pet	6	0/25	(20)
Kanamycin	10	pet	3	0/4	(20)
Neomycin sulfate	20	ND	5 cc	0/22	(19)
	20	pet	4	0/37	(20)
	20	pet	9 cd	0/64	(22)
	1	aq	15 cd	0/28	(21)
Polymyxin B sulfate	20	pet	3	0/6	(20)
	5	ND	1 cc	0/26	(19)
Azithromycin	1	pet	1 cc+cd		(23)
	5	pet			
	10	pet		0/10	
Antiviral drugs					
Idoxuridine	5	dimethyl-acetamide	3 cd	ND	(24)
	1	pet	2 cd	0/12	(25)
	0.5	pet	1 cd	ND	(26)
	0.2	pet	1 cd	ND	(27)
Trifluridine	10 + 5	pet	1 cd	0/50	(28)
	Eyedrops 1%	As is	2 cd	ND	(30)
	Ointment	As is	1 cd	ND	(29)
Beta-blockers					
Carteolol	Eyedrops	As is	1 cd	0/23	(31)
	½	aq	1 cd	0/5	(32), (33)
Betaxolol	1/2/5	aq	1	ND	(34)
Befunolol	1	aq	6 cd	0/20	(35)
Levobunolol HCl	1	aq	1 cd	ND	(36)
Metipranolol	2	aq	1 cd	0/15	(37)
Metoprolol	3	aq	5	0/19	(38)

(*Continued*)

TABLE 17.1 *(Continued)*

Allergen (group)	PT Concentration (%)	Vehicle	PT-positive Cases	PT-negative Cases	References
I-Pentobutolol sulfate	2	aq	1 cd	0/15	(37)
Timolol	0.5	aq	1	0/19	(38)
Timolol maleate	5/1/0.5/0.25	aq	1 cd	0/20	(39)
Non-steroidal anti-inflammatory drugs					
Trometamol	1/0.50/0.10/0.05	aq	1 cc/cd	ND	(40)
Diclofenac	1/2	pet	1	0/10	(41)
Ketorolac	0.5	aq	1 cd (T.R.U.E. Test®)	ND	(42)
Antihistaminics					
Pheniramine maleate	1	aq	1 cc+cd	ND	(43)
Ketotifen fumarate	0.069	aq	1	ND	(44)
Chlorpheniramine maleate	5	pet	1 cd	ND	(45)
Sodium cromoglycate	2	aq	1 cd	0/15	(46)
Olapatadine	0.1	aq	ND	ND	(47)
A2-adrenergic agonists					
Apraclonidine	Eyedrops	As is	1 cc	0/20	(48)
	10	aq	1 cc	0/20	(48)
	1% Eyedrops	As is	31/64 cc/cd	ND	(49)
Brimonidine	0.2% Eyedrops	As is	1 cc	ND	(50
Mucolytics					
N-acetylcysteine	10	aq	1 cd	0/14	(51)
Anesthetics					
Proparacaine	0.5	pet	1 cd	ND	(52)
Tetracaine	1	pet	2	ND	(53)
Benzocaine	5	pet	7 cd	0/70	(24)
	5	ND	1 cc	0/26	(19)
Procaine	5	aq	3 cd	0/40	(21)
Oxyburprocain	0.4	ND	1 cc	0/24	(19)
Proxymetacaine	0.5	aq	1 cd	ND	(21)
Mydriatic					
Dipivalyl epinephrine hydrochloride	0.5	aq	1	ND	(54)
Cyclopentolate hydrochloride	0.1/0.5	aq	1	0/20	(55)
Atropin sulfate	2	ND	1 cc	0/24	(19)
	2 + 1	aq+pet	1 cd	ND	(56)
	0.0006	ND	1 cd	ND	(57)
Epinephrine HCl	1	aq	1 cd	ND	(58)
	1/0.1	aq	1 cd	ND	(59)
d-Epinephrine	1	aq	1 cd	ND	(58)
Homatropine	1	ND	1 cd	ND	(57)
Phenylephrine HCl	10/5/2.5/1	aq/pet	1 cd	0/49	(60)
	10/5/1	aq/pet	1 cd	0/20	(61)
	10	aq	1 cd	0/20	(62)
	5	pet	1 cd	ND	(63)
	1/0.1	pet	1 cd	0/20	(64)
	1	aq	1 cd	ND	(65)
	1	ND	1 cd	ND	(57)
Scopolamine hydrobromide	0.25	ND	1 cc	0/24	(19)
	0.25	ND	1 cd	ND	(24)
Tropicamide	1	ND	1 cd	ND	(57)
Miotic					
Pilocarpine nitrate	2	ND	1	ND	(66)
Pilocarpine HCl	4	ND	1	ND	(66)
Pilocarpine chlorhydrate	4	pet	1	0/12	(67)

Antiseptic					
Resorcinol	1	pet	1	ND	(68)
Interferon					
Beta-interferon	Eyedrops	As is	1	0/10	(69)
Preservatives					
Benzalkonium chloride	0.13	aq	1 cc	ND	(70)
	0.1	ND	3 cc	0/5	(71)
	0.07/0.05	aq	6 cc	0/94	(68)
	0.01	aq	1	0/35	(20)
	0.005	aq	2 cc	0/4	(72)
Chlorhexidine gluconate	1	Ethanol 70%	1 cc	0/14	(73)
	1	aq	3 cc	0/38	(74)
Benzethonium chloride	1	aq	1 cc	0/38	(74)
	0.1	aq	2 cc	0/18	(75)
Cetalkonium chloride	0.01	aq	3 cd	0/18	(21)
Phenylmercuric nitrate	0.05	pet	3 cc	0/17	(75)
Sorbic acid	2.5	pet	3 cd	0/22	(21)
	2	pet	1 cc	0/7	(71)
	0.5	pet	5 cc	0/15	(75)
Thimerosal (Merthiolate)	0.1	pet	126 cc	0/326	(76), (77), (73), (75), (74),
	0.1	pet	39	ND	(60), (78), (79), (80)
	0.1	pet	2 cd	ND	(81), (82)
	0.1	pet	4 cd	0/4	(71)
	0.1	ND	1 cc	ND	(60)
	0.1	ND	1 cc	0/26	(19)
	0.01	aq	1	0/33	(20)
Chlorobutanol	0.5/0.1/0.05	pet	1 cc (Conjunctival provocation test CPT)	ND	(83)
Others					
Bismuth oxide	5/2/0.5	pet	1 cd	0/10	(84)
	Ointment (Noviform)	As is	1 cd	0/10	(84)
Parabens	Eyedrops (Clarvisan)	As is	1 cc+cd	0/25	(85)
	3	pet	1 cc+cd	0/25	(85)
Amlexanox	1	pet	2	0/20	(86)
D-Penicillamine	1, 2.5, 3	aq	2	0/21	(87)
Rubidium iodide	1	pet	1	0/20	(88)
Papaine	1	pet	1 cc	0/19	(75)
	0.1	aq	1	0/10	(89)
Tegobataine L 7	2 + 1	pet+aq	1 cd	0/40	(82)
Epsilon-aminocaproic acid	10/1/0.1	aq	1 cd	0/20	(90)
Tolazoline	10	aq	1 cd	0/3	(91)
Echotiophate iodine	5/2/1/0.25	aq	1 cd	0/20	(62)
Myroxylon pereirae (Peru Balsam)	ND	ND	1 cc+cd	ND	(92)

Abbreviations: PT, patch test; ND, not done; NG, not given; cc, contact conjunctivitis; cd, contact dermatitis; aq, aqueous; pet, petroleum.

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18 Textiles and human skin, microclimate, cutaneous reactions: An overview

Wen Zhong, Malcolm M. Q. Xing, Ning Pan, and Howard I. Maibach

INTRODUCTION

The skin is a large barrier organ that protects the human body from environmental hazards (heat, cold, chemicals, mechanical forces, and so on), and maintains the integrity of the body, while the clothing system provides an extra layer(s) of barrier to enhance the esthetic, thermophysiologic and sensorial comfort of the wearer. However, direct contact and interactions between textiles and skin may cause reactions, even damage or diseases.

This chapter overviews research in the interdisciplinary area of textile–skin interactions and related skin reactions and injuries. First, a brief description relates microclimate in the skin–clothing system, and especially the skin responses to moisture and heat transfer within this system, as this plays a critical role in skin irritation and intolerance caused by textiles. Then follows a discussion on skin irritation reactions to textiles, including dermatitis caused by chemicals (dyes and finishes) and physical contact/friction. Finally, two skin injuries, blisters, and pressure ulcers, which are caused by physical contact, pressure, and friction, are discussed. And the role that textiles play in the prevention and formation of these injuries will be examined.

MICROCLIMATE

The stratum corneum (SC) plays an important role in the clinical appearance of the skin as a result of its water-holding capacity and lipidic content (1,2). From the deeper, highly hydrated layers of the epidermis and dermis, a passive flux of water takes place toward the more superficial SC layers, which have a relatively low water content. This is the so-called transepidermal water loss (TEWL) (3), which is a parameter to evaluate the function of SC as a barrier to prevent excessive water loss.

Extensive research work has been published on the topic of TEWL (4–7); however, knowledge about the influence of textile materials to TEWL is limited.

In 1987, Hatch et al. reported an in vivo study of water content in the surface layers of human SC and water evaporation from its surface due to placement of fabric on skin for varying time periods (8). A lightweight fabric placed on skin produced no change in skin water content or evaporative water loss from the SC. Only for occluded treatments (e.g., fabric plus plastic film), did water content and evaporation increase as the covering materials remained for longer periods.

Another water loss route through skin is via perspiration or sweat, which is secreted by eccrine sweat glands deep in the dermis. Water evaporation from the secretion absorbs heat, and thus helps regulate body temperature in response to environmental changes. For humans to feel comfortable, a fairly narrow surface temperature and humidity must be maintained in the air immediately surrounding the body. Clothing, therefore, plays an important role in regulating body temperature and controlling heat loss. The term microclimate, accordingly, has been used frequently to describe the environmental parameters that influence heat exchanges, such as the temperature, humidity, and microspace air stream between the skin and clothing (9). Microclimate is an important factor for wear comfort, and depends on properties, such as moisture and heat transport through the material, and on physiologic and environmental conditions.

Clothing comfort has been extensively studied; however, less has been done in the skin response to fabric in various conditions. Hatch et al. published on in vivo cutaneous and perceived comfort response to fabric (10–15); this series began with experiments in a simulated skin–fabric–microclimate system, which is composed of a modified Kawabata Thermolabo apparatus housed in a controlled environmental chamber (12). The three experimental fabrics (one cotton and two polyester fabrics with different fiber deniers), showed small differences in water vapor and air permeability as well as energy dissipation rates. The results suggested that these thermophysiologic comfort parameters related more to fabric structures than to fiber contents. In addition, different mechanical and surface properties of fibers may contribute to variation in sensorial comfort of the experimental fabrics (10). They then documented water content and blood flow in human skin under garments worn by exercising subjects in a hot and humid environment (11); no significant differences were observed between the three experimental fabrics in terms of alteration in capillary blood flow, stratum corneum water content, skin evaporative water loss, or skin temperature (13). Surprised by the results, further investigations were performed when fabric patches were placed directly in contact with volar forearm skin of subjects in stead of clothing worn loosely by subjects (14,15). The experiments revealed that the SC hydration reduced after being in contact even with hydrophilic fiber (cotton).

Kwon et al. compared the physiologic effects of the hydrophilic and hydrophobic properties of the fabrics in exercising and resting subjects with and without wind (16). Materials included three kinds of clothing ensemble with different moisture regains (wool–cotton blend with high moisture regain; 100% cotton with intermediate regain; and 100% polyester with low regain). They concluded that the hydrophilic properties of the fabrics studied

were of physiologic significance for reducing heat strain, including skin temperature, clothing microclimate temperature and humidity, and pulse rate, during both exercise and rest, especially when influenced by wind.

Generally, the experiments and analysis on the skin response to textile and clothing system have yet to lead to commercial interventions. This may be caused by the individual differences among human subjects in terms of physical status and sensitivity. When it comes to the in vitro experiments, the difficulties lie in how to realistically represent the whole skin–fabric–microclimate system.

SKIN REACTIONS TO TEXTILES

The skin's irritant reactions to textiles may be caused by chemicals and/or physical contact and friction.

Allergic Contact Dermatitis

Numerous chemicals may be incorporated into the textiles and clothing during the processes from fiber formation, spinning, fabric construction to dyeing and finishing. These chemicals, when in contact with human skin, may cause allergic contact dermatitis (ACD).

Hatch (17) reviewed the occurrence of dermatologic problems caused by consumer exposure to dyes on clothing. Thirty-one dyes, mainly disperse with anthraquinone or azo structures, have been suggested to cause ACD. Subsequently, they reviewed the literature concerning textile dye dermatitis published during the decade before, and four new dye allergens were identified (18). Studies on ACD prevalence, the amount of ACD cases that are presented in a population, were summarized in 2000 (19). Most studies, however, were conducted in Europe, primarily Italy. And all the tests were performed by placing a dye, mostly disperse dye, with unknown purity instead of a dyed fabric directly on the skin.

Accordingly, they adopted the term "textile-dye ACD" in contrast to "color-textile ACD" (20), as the latter case involves more complicated factors, such as dye molecules transferred or released from textiles to the skin, perspiration fastness of the dyes. It was also reported that dyes to which a patient was patch-test positive were infrequently identified in the fabric suspected to be the cause of the skin problems (21). This means that further investigation is desired in the diagnosis and management of colored-textile ACD.

They further reviewed textile chemical finish dermatitis (22). Chemicals used on fabrics to improve 10 performance characteristics have been detected to have resulted in irritant or ACD. The most significant problem is due to formaldehyde and *N*-methylol compounds for durable press fabrics. An updated review on textile dermatitis caused by resins, additives, and fibers ended in 1994 (23). Textile formaldehyde resins for durable press finish was still the focus, as formaldehyde released from the resin was believed to be the causal agent.

Hatch provided a list of those textile chemicals (dyes, finishes, and additives) reported to cause textile dermatitis and the types of fabrics on which these chemicals are most likely present (24). Clinical aspects of textile dermatitis and methods available to identify the specific chemical causing a skin problem are also covered.

However, the extent of the skin problems caused by textile-associated chemicals is hard to define and predict, due to a series of factors, including variation of skin's sensitivity, capacity of absorption and reaction among different people, transfer of irritant chemicals from textiles to skin, and synergy of sweating, pressure, and friction.

Skin Irritation by Physical Contact/Friction

The frictional properties of skin are of interest in the area of cosmetic products and clinical dermatologist dealing with acute and chronic friction trauma, such as blister and callus.

In 1990, a study on frictional properties of human forearm and vulvar skin was reported (25). The dynamic friction coefficient between skin and a Teflon probe was measured in vulvar and forearm skin of 44 healthy female volunteers and its correlation with age, body weight, height, TEWL, and skin capacitance was obtained using multiple-regression analysis. They observed that a higher friction coefficient of vulvar skin (0.66±0.03) than that of forearm (0.48±0.01) may be due to the increased hydration of vulvar skin. And age differences seem to exist for TEWL and friction coefficient in forearm but not in vulvar skin.

A similar study on skin friction properties involved human subjects of different gender and age (26). Measurements were obtained from 11 anatomic regions, namely, the forehead, upper arm, volar and dorsal forearm, postauricular, palm, abdomen, upper and lower back, thigh, and ankle. The dynamic friction coefficient did not vary significantly between age and sex groups but varied considerably among the anatomic regions. They suggested that frictional properties of skin are dependent more on water content or nonapparent sweating and the role of sebum secretion is suggested as one possible factor. And a later study suggested that the surface lipid content plays a limited role in frictional properties of skin (27).

Other studies on the influence of skin friction on the perception of fabric texture and pleasantness under a series of environmental conditions from neutral to hot-dry and hot-humid also revealed that moisture on the skin surface increased skin friction (28) and that fiber type and moisture influenced fabric-to-skin friction measurements (29). These reports agreed on that moisture on the skin is more important than the fiber type or fabric construction parameters in determining the nature and intensity of fabric-to-skin friction and that glabrous skin friction changes less with wetting than with hairy skin.

Recent studies have further investigated the role of moisture, sebum, and emollient products on skin friction properties (30). Elkhyat et al. recorded the influence of hydrophilic–hydrophobic balance of the skin surface on the friction coefficient, using both in vitro and in vivo experiments (31). They showed that the higher hydrophobia tendency of the surfaces, the lower the friction coefficient. The friction coefficient, therefore, may quantify the influence of lubricants/emollients/moisturizers applied to the skin. And the relationship between the friction coefficient and the hydrophilic–hydrophobic balance can be reversed in the presence of water and sebum on forehead.

As to the experimental methods for measuring frictional coefficient of the skin, the earlier designs fall into two categories, using either a probe moved across the skin in a linear fashion (32) or a rotating probe in contact with the skin surface (33,34), as also described in a review article (35). Recently, there are reports about instruments capable of measuring friction coefficient of skin in real time, such as a commercially available UMT series Micro-Tribometer. Either a stainless steel ball (36) or a copper

cylindrical friction/electrical probe (37) was pressed onto the skin with a preset load and moved across the skin at a constantly low velocity. And the UMT continuously monitored the friction force of the skin and the normal force applied by the probe to calculate the friction coefficient in real time. Another commercial device for measuring surface properties of textile materials, a KES-SE Frictional Analyzer (38), was used in skin friction evaluation (39), where the friction coefficient (MIU) and its mean deviation (MMD) were used as the parameters to indicate surface friction. In addition, Tanaka et al. launched a study on a device for monitoring skin conditions, including roughness and softness (40). The device, the so-called Haptic Finger, was designed using PVDF piezopolymer film as a sensory receptor. Signals obtained by sliding the sensor over skin surfaces were processed by wavelet analysis, and the dispersion of the power spectrum density in the frequency domain was obtained and found to be associated with roughness and hardness of skin in both in vitro and in vivo experiments.

However, measurements of the friction coefficient of skin by different devices lack comparability, for there is still disagreement on which scientific law governs the relationship between the pressure and skin friction. The classic Amonton's law (41), which stipulates that the friction coefficient remains unchanged under varying normal loads and speeds of the probe (i.e., the opposing material used to measure the skin friction), was long challenged by numerous research works, including some recent ones (36,39), in which the friction coefficient is found to be inversely proportional to load (42).

Compared with what was achieved in measuring frictional coefficient of skin surface, far less work was performed in the assessment of frictional force between skin and fabric. This usually involved slowly pulling fabric samples across the surface of a subject's skin (i.e., forearm). The frictional force required to pull each fabric across the skin was recorded by a force transducer. The pressure between fabric and skin was often applied by suspending a weight to the free end of the fabric. The resulting irritation effects caused by friction could then be documented (28,29). Other methods for measuring skin–fabric frictions were achieved by using strain gauge (43), or, strained gauged flexure couples, which arranged in a way trying to detect both normal and frictional force (44). Measurements can be made when wiping the material with the right index finger.

Literature concentrating on the skin irritation caused by contact and/or friction of clothing or other textile materials has been summarized by Hatch and Maibach (45). Six fibers that had been reported to be linked to dermatologic problems were covered: nylon, for contact dermatitis and contact urticaria; wool, for acute and cumulative irritant dermatitis, aggravate atopic dermatitis, ACD and immunologic contact urticaria; silk, to atopic dermatitis and contact urticaria; glass fiber, to mechanical irritation; and spandex and rubber fibers. Some dermatitis, such as in the cases of nylon, spandex and rubber fibers, were often caused by dye, finish or fiber additive instead of fiber material itself.

A study on the effects of wearing diapers on skin showed that skin wetness was proportional to diaper wetness, and, with increased skin wetness, there were increased coefficients of friction and increased abrasion damage (46). Studying the electrostatic potentials generated on the surface of the scrotal area, the accumulated electrostatic charges on the pants were due to the friction of the pants with the skin, when different types of textile fabrics were worn (47). The polyester pants showed the highest potential while the polycotton pants produced less than half that level. The readings at day time were higher than at night, probably due mainly to the higher temperature and activities during the day. A related study even suggested that this electrostatic potential may be responsible for inhibiting hair growth (48).

In an effort to develop test methods to evaluate certain consumer products, such as feminine hygiene products and diapers, for their potential in causing mechanical irritation during use, Farage et al. investigated several test sites on the human body where normal daily activities provided the opportunity for movement and therefore friction (49). These studies indicate that a protocol using the back of the knee as a test site with an exposure regimen of 6 hours daily for four days, proved to be the most effective test system for evaluating mechanical irritation.

Prolonged or extensive contact combined by pressure, friction, or shear between fabric and skin may lead to more serious problems or injuries, such as friction blisters and pressure ulcers, as discussed in the next section.

SKIN INJURIES

Friction Blisters

Friction blister is a frequently occurring skin problem associated with sports and vigorous activities. It can be critical if they occur during athletic competitions or military missions, when reduced performance or mobility becomes costly, injurious, or fatal. Accordingly, extensive research has been performed on the blister—causing fabric–skin friction and interactions.

Studies showed that blisters result from frictional forces that mechanically separate epidermal cells at the level of the stratum spinosum. Hydrostatic pressure causes the area of separation to be filled with a fluid that is similar in composition to plasma but has a lower protein level (50).

There were a series of reports on a specially designed apparatus for producing friction blisters on human skin in late 1960s and early 1970s (51–55). The instrument consisted of a rubbing head to which various materials (including textiles) could be firmly attached. The head could be moved over the surface of any chosen skin site at a selected stroking rate under a known amount of load. Frictional coefficient and temperature could also be recorded. Observations (54) and healing treatments (52) were performed on blisters formed by the instrument on human volunteers.

The other studies on friction blister (mostly foot blisters) formation and prevention were usually performed by recording the prevalence and size of blisters among a group of subjects with routinely heavy load of activities, such as athletes or military personnels (56–59).

For example, Herring and Richie conducted a double-blind study to determine the effect of sock fiber composition on the frequency and size of blistering events in long-distance runners (57). Socks were tested, which were identical in every aspect of construction except fiber composition: One was composed of 100% acrylic, and the other 100% natural cotton fibers. Acrylic fiber socks were associated with fewer blistering events and smaller blisters compared with cotton fiber socks.

Another examination into the effect of sock fiber contents on the incidence and severity of foot blister was reported by Knapik et al. (59). Three hundred fifty-seven military trainees were divided into

three groups and assigned one of the three boot-sock system: the standard military boot sock consisting of a wool–cotton–nylon–Spandex combination; the standard military boot sock with a thin inner or liner sock consisting of polyester; a very thick, dense, prototype outer sock consisting of a wool–polypropylene combination over the same liner sock as the second group. The standard military sock with a polyester liner reduced the incidence of severe blisters, but the dense sock with the polyester liner reduced the overall incidence of blisters as well as the incidence of severe blisters.

Patterson et al. studied the blister attach rate among 100 cadets in a summer camp (58). Studies showed that women had higher risk than men. Cadets with a history of blisters in the two years before camp had an increased relative risk of blister formation. It was also suggested that the foot should be preconditioned to its footwear to prevent blister formation, say, wearing the boots over 20 hours per week during the two weeks immediately before camp.

Other measurements to prevent blister formation include lubrication (60), decreasing friction/shear (61), or reduce the skin surface hydration as moisture skin increase frictional force (50). However, very dry or very wet skin would decrease frictional forces.

Reynolds et al. investigated the influence of an antiperspirant with emollient additives on frequency and severity of frictional blisters, hot spots, and irritant dermatitis by having 23 healthy subjects walking on a treadmill in a warm environment (62). However, the results showed that it reduces irritant dermatitis but does not reduce foot-sweat accumulation, blister or hot spot incidence, or blister severity. A later study was carried out on the effect of an antiperspirant in reducing foot blisters during hiking (63); it might be effective in reducing foot blisters during hiking; however, a side effect of skin irritation was observed.

Despite extensive studies on friction blisters, the prevalence or severity of friction blister is still difficult to predict, let alone a simple solution to prevent its formation. The cause may lie in the dramatic variation of skin conditions (surface roughness, hydration, adhesion between skin layers, and so on) among individuals as well as among different anatomic sites of the same person.

Pressure Ulcers

Pressure ulcer, defined as an area of localized damage to the skin and underlying tissue caused by pressure, shear, friction, or a combination of these (64), presents a significant health care threat to hospitalized patients. Approximately 1 million hospitalized and nursing home patients are diagnosed with pressure ulcers and about 60,000 die as a result of pressure ulcer complications annually (65). Related costs have been estimated to exceed $1 billion annually in the United States (66,67).

According to etiology of pressure ulcer formation, when compressive and/or shear/friction forces reach certain threshold (combination of intensity and duration), there is occlusion and thrombosis of capillaries at pressure points or areas. This results in tissue anoxia with release of toxic metabolites, and ultimately cell death and tissue necrosis. Pressure ulcers are thus formed (64,68).

As the principal mechanical factors intriguing ulcer formation, the combination of pressure and shear/friction has been reported to be devastating to the skin and underlying tissues. Dinsdale demonstrated that when both pressure and friction were applied to the skin of swine, a pressure as low as 45 mmHg was sufficient to cause an ulcer, while 290 mmHg of pressure was required if no friction was present (69). Davis presented hypotheses of three scenarios with different shear and vertical force conditions that could lead to skin ulceration (70): at a localized area the skin may tend to slip (*i*) toward, (*ii*) away from, or (*iii*) parallel to a neighboring skin region where the two skin regions possess different friction coefficients against the slippage.

Despite all the different scales for assessing pressure ulcers, there are some common factors that are included or considered (71,72), namely, pressure, shear/friction, and liquid/moisture. Among the overwhelming publications in pressure ulcers research, including updated reviews (59,73–75), only a few have been devoted to the role that textiles play in the formation and prevention of pressure ulcer (76), although textiles could interfere with all the following important factors associated with pressure ulcers.

Pressure

Although the fabrics (clothing and beddings) alone cannot do much to reduce the pressure experienced by patients (other solutions, such as repositioning, using pillows/cushions/foam wedges, or using low-pressure mattress, or seats that can better perform the job (77–79)), it would play a critical role in governing the shear and friction actions on human skin once pressure and body motion are involved.

Nevertheless, there have been studies on specially designed clothing/socks in terms of their effectiveness in prevention and management of pressure ulcers. For example, padded hosiery has been reported to reduce plantar pressures in patients at risk for ulceration (80). Specially designed socks, when worn with suitable shoes, may be an acceptable and inexpensive addition to existing methods of protecting the high-risk insensitive diabetic foot ulceration (81).

Shear Stress and Friction

The surface smoothness of fabrics and stiffness/flexibility of fiber and fabric may be two of the important factors in determining the shear and friction experienced by patients. Little has been done on the effort of shear/friction monitoring in preventing pressure ulcers. Snycerski and Frontczak-Wasiak presented a design and manufacture of a double-layer woven fabric for bed sheet with different friction coefficients on both sides of the fabric (82): the bottom side of the bed sheet has a higher friction coefficient so as to limit the slip between bed sheet and underlying bedding materials, and therefore reduce bed sheet wrinkling; the upside of the bed sheet has a low friction coefficient to allow easier and smoother position change for patients. However, the efficacy of this sheet in controlling pressure ulcers has not been reported.

Liquid/Moisture or Skin Hydration

Appropriate moisture conditions should be kept to prevent or reduce ulceration. An over dry condition may lead to a skin more vulnerable to cracking. Conversely, a wet condition (because of incontinence and/or perspiration) may cause skin maceration and lower the tissue tolerance to shear stress and friction (68). It may also create a favorable condition for the growth of microorganisms.

The clothing (and the bedding) system plays an important role in moderating liquid and moisture so as to maintain a healthier microclimate near the skin surface.

The role of textiles play in the formation and prevention of pressure ulcers is generally understudied, despite that textiles (clothing and bedding) could have considerable influence on the factors (pressure, shear/friction, and skin hydration) contributing to skin ulceration. More research effort, therefore, is expected in this field for a better understanding as well as a more efficient way in controlling the problem.

SUMMARY

Skin provides the critical first defense mechanism for the body in dealing with external hazards. Clothing fabrics and the skin surface constitute a buffering system that establishes a thermal and sensorial state of comfort to maintain human health and normal functions. A failure of fabric–skin regulatory interactions can lead to various problems, from thermophysiologic discomfort, irritation, to injuries, such as blisters and pressure ulcers.

We reviewed here the existing studies in the fabric–skin interactions, related irritation reactions, and injuries. The microclimate between clothing and skin surface, where fabric–skin interactions take place, has been discussed. Skin irritations caused by both textile chemicals and physical skin–textile contact/friction have also been reviewed. The final section deals with skin injuries, blisters, and pressure ulcers, caused by physical contact, pressure, and friction.

Despite the prevalent problems caused by ill textile–skin interactions, few research efforts have been devoted to this field. In addition, the existing in vivo experimental studies have rarely led to any significant results and solid conclusions. The cause may lie in the dramatic variation of skin conditions (surface roughness, hydration, adhesion between skin layers, and so on) among individuals as well as among different anatomic sites of the same person. Another reason might be the lack of communication between researchers in the areas of textiles and dermatology.

APPENDIX: LITERATURE SUMMARY

Topic	Results	References	Comments
Microclimate			
TEWL of human skin		(1–7)	Dermatology studies
Microclimate		(9)	Concept
In vivo cutaneous and perceived comfort response to fabrics:	Light weight fabrics produced no changes Thermophysiologic comfort parameters related more to fabric structure than to fiber content	(8,10–15)	Individual differences among human subjects in terms of physical status and sensitivity.
Water content and evaporation on human skin surface			
Experiments in simulated skin–fabric–microclimate system	No significant differences between experimental fabrics SC hydration reduced when in contact with hydrophilic fiber		Has yet to lead to commercial interventions
Mechanical and surface properties of fibers on sensorial comfort			
SC water content, evaporation, and capillary blood flow in hot and humid environment			
Patch test on volar forearm skin			
Physiologic effects of hydrophilic properties of fabrics	Hydrophilic properties of fabrics were of physiologic significance for reducing heat strain when influenced by wind	(16)	
ACD			Extent of the problems hard to define and predict, due to variation of skin sensitivity, capacity of absorption and reaction, transfer of irritant chemicals from textiles to skin
Textile dye dermatitis	Review on identified dyes that cause skin problems, prevalence	(17–19)	
"Textile-dye ACD" and "Color-textile ACD"	Dyes to which a patient was patch-test positive were infrequently identified in the fabric suspected to be the cause of skin problem	(20,21)	
Chemical finish dermatitis	Review on identified chemicals that causing skin problem	(22–24)	
Skin irritation by physical contact/friction			
Frictional properties of human skin	Frictional properties varies among human anatomic regions	(25–27)	Prolonged or extensive contact combined by pressure, friction, or shear between fabric and skin may lead to more serious problem or injuries, such as friction blisters and pressure ulcers
Moisture on skin friction	Moisture on skin more important than fiber type in determining frictional properties	(28,29)	
Moisture, sebum, and emollient on skin friction	The higher hydrophobia tendency of the surfaces, the lower the friction coefficient	(30,31)	

(*Continued*)

Topic	Results	References	Comments
Measuring skin friction:	Measuring friction coefficient in real time	(32–35,37–40)	
Probe moved across skin in linear fashion	Recording friction coefficient and its mean deviation		
Rotating probe	A sensory receptor		
UMT series Micro-Tribometer			
KES-SE Frictional Analyzer			
"Haptic Finger"			
Skin friction versus load:	Friction coefficient unchanged with varying normal loads	(36,39,41,42)	
Classic Amonton's law	Friction coefficient inversely proportional to load		
Challenged by recent work			
Assessment of frictional force between skin and fabric	Frictional force required to pull each fabric across the skin recorded by a force transducer	(28,29,43,44)	
	Frictional force measured by strain gauge or strained gauged flexure couples		
Skin irritation caused by textile fiber contact/friction	Review on identified fibers that cause skin problem	(45)	
Skin irritation by diaper, feminine hygiene products	Increase friction and abrasion with increased skin wetness	(46,49)	
Electrostatic potentials generated by fabric–skin friction	Polyester pants showed highest electrostatic potential	(47,48)	
Friction blister			
Blister formation		(50)	Prevalence or severity of friction blister difficult to predict, let alone a simple solution for prevention. This cause may lie in the dramatic variation of skin conditions among individuals and among different anatomic sites of the same person
Special apparatus for producing friction blisters on human skin	Observations and treatments performed on blisters formed by the instrument on human skin	(51–55)	
Blisters, studies on prevalence		(56–59)	
Blister prevention:	Acrylic socks associated with fewer blistering than cotton	(50,57,59–63)	
Design of socks	Antiperspirant might be effective in reducing foot blister		
Lubrication			
Decreasing friction/shear			
Reducing skin surface hydration			
Pressure ulcers			
Pressure ulcer, prevalence, and cost		(64–67)	Role of textiles play in the formation and prevention of pressure ulcers is generally understudied, despite that textiles (clothing and bedding) could have considerable influence on the factors (pressure, shear/friction, and skin hydration) contributing to ulceration.
Pressure ulcer formation:		(64,68)	
Etiology		(70)	
Hypothesis on shear and vertical force conditions			
Principal mechanical factors:	Most important factor	(71,72)	
Pressure	The presence of shear significantly reduce the threshold of pressure that intriguing ulcer formation	(77–79)	
Shear/friction		(69)	
Skin hydration		(68)	
Ulcer prevention, design for textile materials:	Padded hosiery may reduce planar pressure in patients at risk of ulceration	(76)	
Pressure reduce (socks)		(80,81)	
Friction control (bedding)		(82)	

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19 Identifying the source of textile-dye allergic contact dermatitis: A guideline

Kathryn L. Hatch and Howard I. Maibach

When a dermatologist concludes that the cause of a patient's allergic contact dermatitis (ACD) is a fabric dye, two major challenges follow: (*i*) to assist the patient in identifying the offending textile product(s); and (*ii*) next to provide directions to the patient so that he/she can avoid the purchase of skin contact textile products that will cause a recurrence of his/her ACD skin lesions. Explaining how to identify the offending product(s) and to purchase textile products that will not cause a recurrence of skin lesions can be daunting because textile products are rarely labeled to reveal their colorants. About the only fabric "ingredient" revealed on textile labels is fiber content, information required by many countries including the USA, European Union countries, Japan, and South Korea.

Because many dermatologists are unsure about what directions to provide patients and the literature revealed no written procedures, our objective is to provide a guideline for identifying which textile products/fabrics owned by patch test–positive patients are the most likely to contain the dyes to which each is patch test positive. We present this procedure in such a manner that patients can also use it to avoid the purchase of skin contact textile products that are most likely to cause a recurrence of their ACD skin lesions. The procedure involves four sequential steps.

IDENTIFY ALL COLORED TEXTILE PRODUCTS THAT CONTACTED THE AFFECTED SKIN AREA

The first step is for the patient to collect or list all colored-fabric textile products that contact the skin area where his/her skin lesions appear. It is important not to eliminate potential ACD allergen-containing items during this first step. Patients need to think about the various locations where garments are kept to consider all items that touch their skin where lesions appear.

To assist in the listing or rounding-up task, we include Table 19.1 which provides a checklist of categories of textile products that contact various skin areas. During this process each patient should: (*i*) consider all garments, bed linens (sheets, pillowcases, comforters, blankets), and other household textile products that contact their skin (towels, carpets, and rugs, and upholstered furniture and car seats), (*ii*) include skin contact products made from solid-colored fabrics, including black (which some would argue are not colored fabrics) and those made from multicolored fabrics, (*iii*) include long-time use or frequent-use items, because these are as likely or even more so to contain the ACD allergen as "newly acquired" items due to the length of time that dye allergens need to cause sensitization, and (*iv*) include those outerwear garments that contact skin areas where lesions appear. An outwear garment with a collar might be the cause of neck area lesions, with sleeves longer than garments worn underneath might cause lesions on arms, wrists, or hands, and/or with pockets might cause lesions on the hands.

NARROW NUMBER OF SUSPECT ITEMS USING FIBER COMPOSITION AND PATIENT PATCH TEST RESULTS

The second step begins with finding the fiber composition of each item collected or listed. Fibers are the fundamental material unit in any fabric; they are the basic "ingredient." Information on fiber composition is most frequently located on a sewn-in label, often along with care information (laundering or dry-cleaning instructions) inside most garments, and along the hems of bed linens and towels. Garments that usually do not have fiber content information attached to them are women's hosiery as well as men's, women's, and children's socks. In these cases, the patient has to recall what the stated fiber composition was on the packaging at the time of purchase.

The reason for finding fiber composition information for each item is that it is the best information available from which one can reasonably deduce the colorant class or classes from which the colorant(s) on the fabric belong(s). As shown in Table 19.2 by the use of X in those locations where a fiber can be dyed with dyes named in the table header row, no fiber can be dyed with every type/class of dye. For example, cotton fibers can be colored with dyes in the direct, reactive, azoic, vat, sulfur, mordant, and pigment classes, but they cannot be colored with dyes in the basic and disperse classes and rarely with dyes in the acid class. Polyester fibers can be colored with dyes in the disperse and pigment classes but not with dyes in other classes. Furthermore, as shown in Table 19.2 by the use of X-mod, certain fibers can be modified to accept dyes in classes other than the "usual" classes. For example, polyester fiber when modified accepts basic dyes and spandex, a fiber always combined with another one to make a fabric and usually dyed with disperse dyes, can be modified to accept dyes in the acid class and in the direct class.

Our recommendation is that each patient sort the textile items in his/her suspect pile or list by whether the item is made from a one-fiber content fabric, using two classes of fiber in the fabric, or made with two or more fabrics, each fabric in the item having a different fiber composition. Examples of items in the latter group would be lined garments, such as coats, suit, and outerwear jackets, tailored dresses, and slacks because the lining "inner fabric" usually has a different fiber composition than the "shell" or outer fabric.

TABLE 19.1
Product Categories to Consider Based on Location of Lesions[a]

Upper Torso	Lower Torso
Back/Chest/Axillae	***Stomach/Buttocks***
Dresses	Pants/trousers
Shirts/blouses	Dresses
T-shirts	Thermal underwear
Undershirts	Slips
Slips	Underpants/drawers
Shapewear	Warm-up suit bottoms
Warm-up suit tops	Pajamas
Pajamas	
Thermal underwear	
Sweaters	
Swimwear	
Athletic wear	
Arms	
Garments listed above with sleeves	
Neckline	***Legs***
Garments listed above with collars	Socks
Jackets/coats with collars	Hosiery
Scarves (decorative and functional)	Dresses
	Skirts
	Pants/trousers
Head	***Feet***
Hats	Socks
Hat bands	Hosiery
Sheets	Sock liners
Pillowcases	Shoes with fabric lining
Blankets	
Decorative pillows	

Note: [a]no upholstery fabrics are included in above table. No evidence to date that upholstery fabric is a source of an allergic contact dermatitis allergen.

Then, the single-fiber fabric group should be divided into a 100% cotton group, a 100% rayon group, which will include garments made from 100% bamboo because bamboo fibers differ from rayon fibers only in the source of the cellulose (bamboo stalks rather than trees) (1), a 100% acetate and triacetate fabric group, a 100% polyester group, a 100% nylon group, a 100% silk group, a 100% wool group, and a 100% olefin (polypropylene) group. There will be no 100% spandex group or 100% rubber group as these fibers comprise only a small proportion of the weight of any fabric. The multifiber fabric items should then be divided into (*i*) a polyester/cotton blend group, (*ii*) those containing spandex, and (*iii*) a miscellaneous group. No subdividing is needed at this time in the multifabric group.

Now *all dye-positive patients* can remove from their suspect group those items (*i*) whose fiber composition is 100% cotton, 100% flax (linen), 100% ramie, 100% rayon (viscose), 100% bamboo, and 100% lyocell, (*i*) made of fabrics with any combination of these fibers, (*iii*) made of spandex and cotton fabrics, and (*iv*) made of 100% polypropylene (Fig. 19.1). The cellulosic-fiber items can be removed as suspects because they may only be colored with colorants from the direct, vat, azoic, sulfur, mordant, pigment, and reactive classes. Within the six first named colorant classes only three colorants, among the thousands of colorants in these classes, have been identified as ACD allergens (2,3). By name those dyes are Direct Orange 34 (CI 40215), Direct Black 38 (CI 30235), and Vat Green 1 (CI 59825). With regard to the reactive dyes, many have been identified as ACD allergens (2,3). However, once applied to fabric they cannot be the cause of ACD because they are covalently bonded to the cellulose polymers and any excess dye on a fabric has a valence state, which destroys its ability to be an ACD allergen. The cellulosic-fiber and spandex combination fabrics can be removed from the suspect list because the spandex in these fabrics is not dyed. Polypropylene items can be removed as suspects because when colored the disperse dyes are added to the fiber spinning solution trapping the dues within the fiber. In other words, there is no disperse dye available for transfer to the skin.

As shown in Figure 19.1, patients who are patch test positive to *disperse dyes only* can remove items having the following fiber compositions from their suspect list: 100% wools (including cashmere, mohair, angora, camel's hair, and alpaca), 100% silk, and 100% acrylic. The fabrics made of wool (including the specialty wool fibers), silk, acrylic, and combinations of these fibers are removed as suspects because they are not dyed with disperse dyes (Table 19.2). Items containing polyester fiber with spandex may be eliminated as suspects, although dyed with disperse dyes because such fabrics are given a "reductive clear," a process that removes disperse dye on the fiber surfaces, leaving all disperse dye molecules embedded within the fibers, thus not available for transfer to the skin. Items that disperse dye-positive patients must keep on their suspect list are those made entirely or partially from (*i*) polyester fibers/fabrics, (*ii*) acetate (cellulose acetate) fibers/fabrics, (*iii*) triacetate fiber/fabrics, (*iv*) nylon fiber/fabrics, and (*v*) combinations of these fibers.

Disperse dye-positive patients are likely to have a considerable number of suspect items because polyester fiber is a component in many fabrics: formal and casual dresses, slacks, blouses, and T-shirts; washable uniforms for nurses, waitresses, and mail and package delivery personnel; fleece jackets (e.g., Polartec®), and bed linens (sheets, pillowcases, coverlets). Acetate (cellulose acetate) is a popular lining fabric in lined jackets and slacks and in fancy/formal dresses. Triacetate and nylon is a fiber combination often in fabrics for women's intimate apparel and sleepwear.

As shown in Figure 19.1, patients who are patch test positive to *acid dyes only* can remove the items having the following fiber contents from their suspect list: (*i*) 100% polyester, (*ii*) 100% acetate, (*iii*) 100% triacetate, (*iv*) combinations of these fibers, and (*v*) polyester blends, such as polyester/cotton, polyester/rayon, and polyester/wool. These fabrics will not contain acid dyes (Table 19.2). Items that must be kept on the suspect list are those made partially or entirely from (*i*) wool (sheep wool), (*i*) specialty wools (angora, cashmere, so on), (*iii*) silk, (*iv*) nylon, and (*v*) combinations of these fibers because these are fabrics that are most likely to contain acid dyes. Wool/spandex, silk/spandex, and nylon/spandex fabrics will contain acid dyes because the spandex in these fabrics will be the type modified to accept acid dyes.

Items likely to be included in the suspect list of the acid dye patch test-positive patients are (*i*) sweaters, business suits, tailored dresses, and dry-cleanable uniforms (military, band, airline pilot) because such items are often made from wool and specialty wool fabrics, (*ii*) expensive blouses, dresses, suits, scarves, women's

TABLE 19.2
Pairing Colorant Class to Fiber Classes

Fiber Groups Based on Origin and Polymer Chemistry[a]	Class Names[b]	Acid	Basic	Disperse	Direct	Reactive	Azoic/Vat/Sulfur/	Mordant	Pigment
Fibers composed of cellulose polymers	Cotton	Some[c]			X	X	X	X	X
	Cotton/Baumwolle								
	Flax (linen)				X	X	X		
	Ramie					X			
	Rayon/viscose/ bamboo				X	X	X		X
	Lyocell				X	X	X		X
Fibers manufactured with chemically modified cellulose	Acetate (cellulose acetate)			X					X
	Triacetate			X					
Fibers composed of protein or polyamide polymers	Wool	X			X	X		X	X
	Cashmere	X			X				
	Mohair	X							
	Angora	X							
	Camel's hair	-----							
	Silk/Soie	X			X	X			X
	Nylon/polyamide	X	X-mod	X-mod[d]	X	X			X
Fibers composed of synthesized polymers (the manufactured synthetic fibers)	Polyester		X-mod	X					X
	Acrylic	X-mod	X						X
	Polyacrylonitrile								
	Olefin/polypropylene			X					
The manufactured synthetic stretch fibers[e]	Spandex/Elastane	X-mod		X	X-mod				X
	Rubber	—	—	—	—	—	—	—	—

Note: [a]The origin of fibers is either from nature or from manufacturing. Natural fibers are produced by plants and animals. Manufactured fibers are derived by a process of manufacturing from any substance, which at any point in the manufacturing process, is not a fiber. Manufactured fibers made from synthesized polymers from the manufactured synthetic class.

[b]The fiber class names shown include those required in U.S.A. and other countries. A manufacturer may elect to use several class names for the same fiber so the product will meet the labeling standards of several countries.

[c]A number of acid dyes will color cellulosic fibers but acid dye is not usually the dye of choice. Even in the dyeing of combination fabrics of silk, wool, or nylon with cotton, the cotton is dyed with direct dye and the other fibers with acid dye.

[d] Mod indicates that it is a modified form of the fiber that can be colored with this dye.

[e] Rubber fiber may also be manufactured from natural rubber that is obtained from rubber trees.

Source: Compiled from Refs. (5–7).

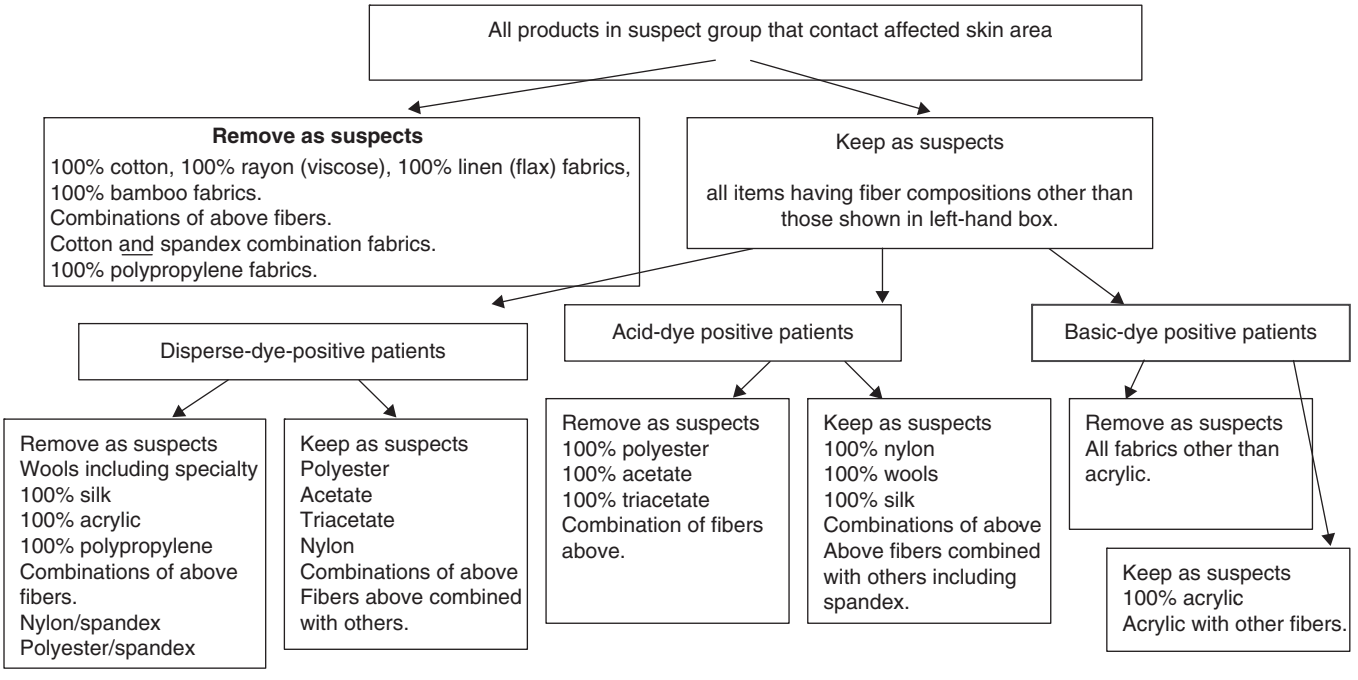

FIGURE 19.1 Guideline for determining which items are most likely to be the source of a patient's allergic contact dermatitis allergens.

underwear, and nightwear, adult-sized thermal underwear, and sheets as these are often made using silk fabrics; and (*iii*) women's sheer hosiery, men's socks, and windbreaker jackets as these items are often made from nylon fabrics.

Patients who are patch test positive to *basic dyes only* can remove most items from their suspect list because basic dyes are only used to dye 100% acrylic fabric and fabrics containing acrylic fiber along with either polyester or nylon fiber. As shown in Table 19.2, polyester fiber and nylon fiber are modified to accept the basic dyes. These modified fibers are combined with acrylic to make the blended fiber fabrics. Items most likely to remain on the suspect list will be blankets, throws, sweaters, socks (especially sport socks), thermal underwear, mitten, gloves, winter-type scarves, and sweat suits.

Not shown in Figure 19.1 are the "keep" and "remove" lists for patients with positive patch tests to dyes of more than one type. These patients can determine their listing by combining "keep as suspect" list in Figure 19.1. For example, patients who are patch test positive to dyes in the disperse- and acid-dye classes will combine the items shown in "keep as suspects" under the dispersedye-positive patient heading: and those in the "keep as suspects" list under the acid-dye-positive patient heading.

CONSIDER THE DYE FASTNESS OF EACH SUSPECT ITEM

The third step is to consider the dye fastness of each item in the suspect group. Dye fastness is a measure of the ability of the fabric to retain dye molecules under various conditions—when the fabric is rubbed (called wet crock fastness when the fabric is moist and called dry crock fastness when the fabric is dry), when the fabric is saturated with perspiration (called perspiration fastness), and when saturated with water (called wet fastness or wash fastness). Generally, fabrics with poor dye fastness when purchased are more likely to be a culprit than fabrics with good dye fastness at the time of purchase. The phrase "at the time of purchase" has been used because dye fastness changes (usually improves) as a textile product is used, because dye molecules may be lost during laundering, as the fabric is abraded (rubbed) and as dye molecules are destroyed by the sun, ozone, and other agents. Therefore, over time less dye is available for transfer to the skin. Considering dye fastness does not allow items to be removed from the suspect list but allows items to be grouped as "more likely suspects" and "less likely suspects."

Wet Fastness and Color Bleeding

A good place to start is to recall whether any fabrics on the suspect list caused the laundry water to become colored, caused other items in the same wash load in which they were included to be stained, or one observed that color "bleed" from one area of the fabric onto another. If there are such items, they should be placed high on the suspect list as there was/is dye available for skin transfer. Then, care labels on the items in the suspect list should be read. When the care label states "Wash Separately" or "Wash with Like Colors" that indicates potential poor color fastness. Such items should also be placed high on the suspect list.

Fastness to Rubbing

Often we do not observe that colorant is being lost when a fabric is rubbed. Fastness to rubbing is important because fabrics continuously rubbed against the skin when in use. A quick test to determine the availability of dye to transfer is to rub the suspected fabric with a white swatch of cotton fabric. Hold the white swatch over the index finger and place the colored fabric on a horizontal surface. Have someone hold the colored fabric, while you rub the surface with the white fabric. Bear down and move index finger back and forth about 10 times. Note whether any dye has transferred to the white swatch (the white cloth is now colored). (This simulates what is known as a dry crock test.) Then wet the white fabric, place over your index finger in another location of thewhite cloth, and again rub the colored fabric. (This simulates the wet crock test). Again assess whether dye transferred (whether the

white fabric became colored). Fabrics with poor crock fastness should be placed in the "more likely culprit" group and those with good crock fastness in the "less likely" suspect group.

Fastness to perspiration: In a laboratory, fastness to perspiration is done by placing the colored fabric in an artificial perspiration solution and then placing this swatch in contact with a white cotton swatch. The assembly is placed in an oven. On completion, the white fabric is observed to determine whether there has been dye molecule transfer. This test is difficult to simulate but in its place, one can recall when an item in the suspect list caused an undergarment to be stained with color in the underarm area, the interior elbow, or at a waistline. These items should be placed in the "more likely" category.

EXAMINE THE COLOR (HUE, SHADE, INTENSITY) OF THE FABRIC

The fourth step in the process can be to sort the fabrics within the most and less likely groups by hue (color) so that the blue and black fabrics are at the top of the suspect list. The rationale for such a ranking is that black and blue fabrics often contain the blue and red dyes that are ACD allergens.

It is also possible to rank items on the suspect list by shade placing those items that are a dark shade of any hue (red, green, blue) higher on the suspect list than those of a light (pastel) shade. The rationale behind the ranking is that a higher concentration of dye molecules is required to achieve a dark shade of any hue and that the concentration of dye available for transfer is critical in determining whether sensitization and/or elicitation ever occur.

SUMMARY AND CONCLUSION

Identifying which textile items most likely contain the colorant to which the patient is patch test positive is not an easy task, but one that can be accomplished by following the four major steps outlined in this article:

1. Identify all colored textile products that contacted the affected skin area;
2. Narrow the number of suspect items using fiber composition and patient patch test results;
3. Consider the dye fastness of each suspect item; and
4. Examine the color (hue, shade, intensity) of the fabric.

The only way to know which fabrics contain the culprit dye (the dye to which the patient is patch test positive) is to send the most likely items to a laboratory for dye content analysis or patch test with a swatch from each of the suspected fabric. Both methods result in rendering the garment/product unusable as the item will be cut to obtain fabric for the test. Nevertheless, this is encouraged. Dermatologists who suspect a patient has a colored-fabric ACD are encouraged to contact the authors of this chapter for possible submission of the suspect garment for dye content analysis. This data will be added to an ongoing study involving the identification of disperse dyes in the fabrics of patients who are patch test positive to at least one disperse dye (4).

An additional step that might be taken after the fabric is known to contain a dye to which the patient was patch test positive is to patch the patient with a swatch of that fabric or with dyes extracted from that fabric. Our recommendation at this time is to wait until there are (*i*) a collection of garments that do contain the allergen to which patients are patch test positive, and (*ii*) a written protocol to conduct the tests (patch with fabric and patch with dye extracts). Then it should be possible to develop reliable procedures.

Patients may use the fabric fiber-composition listings given in Figure 19.1 in the appropriate "remove as suspect box" to assist in purchasing textile items. Additionally they may also look for the Oeko-tex label on garments. This label identifies garments that do not contain most of the known textile dye allergens.

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20 Trichloroethylene dermatotoxicology: An update

C. L. Goh

INTRODUCTION

Trichloroethylene (TCE) is an alkenyl halide and chlorinated hydrocarbon that is commonly used as a solvent in the metal and electronic industry for more than 50 years. TCE has been reported to cause health hazards, including skin eruptions and systemic toxicity, for example, central nervous system, liver, kidney, and the heart. TCE is also used in many other industrial processes, for example, dry cleaning and flavor extraction processes.

The chapter reports on the chemistry and dermatotoxicology of TCE.

PROPERTIES OF TCE

Chemistry and molecular formula: TCE is a halogenated aliphatic hydrocarbon, halogenated alkene, haloalkene, trihaloalkene, chloroalkene, and trichloroalkene with the chemical structure seen in Figure 20.1.

TCE is also referred to as 1,1,2-trichloroethene, 1,1-dichloro-2-chloroethylene, 1-chloro-2,2-dichloroethylene, acetylene trichloride, trethylene, triclene, tri, trimar, and trilene.

Its molecular formula is C_2–H–Cl_3. Its structural formula is Cl_2C=CHCl.

TCE is a highly volatile, clear, colorless liquid with a sweet, chloroform-like ethereal smell. It is easily evaporated and inhaled systemically in a warm environment. It shows high solubility in adipose tissue and is slowly eliminated from the body with a long biological half-life.

USES OF TCE IN INDUSTRY

TCE is an effective solvent for a variety of organic substances. It was initially used to extract vegetable oils from plant produces, decaffeinating coffee and in flavor extraction, for example, from spices. Later it was also used as a solvent in dry cleaning processes.

TCE was widely used as a degreaser for metal parts and refrigerants but in the late 1950s it was largely replaced by 1,1,1-trichlorethane, which is considered less toxic. Over the years, TCE made a comeback because 1,1,1-trichloroethane production has been phased out in most countries under the terms of the Montreal Protocol in the late 1980s to prevent ozone depletion. TCE has experienced a resurgence in use since.

TCE METABOLISM

Because TCE is highly volatile, its vapor is the most frequent form of occupational exposure. The vapor is readily inhaled and 70% of the inhaled vapor is absorbed systemically.

In the body, TCE is mainly metabolized by cytochrome P450 (CYP) to chloral hydrate, which is further converted by alcohol (ADH) and aldehyde dehydrogenases (ALDH) to trichloroethanol and trichloroacetic acid (TCA), respectively(1)). Most of the tricholoroethanol is conjugated with UDP gluconyltransferase to form urochloral acid, some of which is converted by microsomal alcohol oxidation enzyme to TCA via chloral hydrate (2).

Figure 20.2 shows the metabolic pathways of TCE when it is absorbed into the human body. Fifty percent of the absorbed TCE is passed out in the urine as metabolites namely, trichloroethanol (33%) and TCA (18%). Nine percent is exhaled unchanged in the breath and 8% excreted in the feces and sweat.

MECHANISMS IN TCE DERMATOTOXICOLOGY AND TCE HYPERSENSITIVITY SYNDROME

Systemically absorbed TCE can have toxic effects on the neurologic, hepatic, renal systems, and the skin. Many of the toxic effects of TCE are believed to be mediated through immunologic mechanism (3).

It remains unclear how TCE causes generalized skin reactions and hepatitis, which is the most common way TCE toxicity presents to the dermatologists. TCE inhibits the activity of ALDH and the metabolism of low-molecular weight aldehyde with short carbon chains (4). Hence aldehyde may easily accumulate in the body after exposure to TCE. It is hypothesized that this inhibition of ALDH might be the trigger for the generalized skin reactions.

In one study, no serious liver damage was observed in rats exposed to high or prolonged exposure level of TCE (5). However, when CYP isozymes were induced with alcohol, severe transaminitis occurs after exposure to high levels of TCE and serious liver damage occurs. Such induction is not seen with chloral hydrate. It would appear that intermediate metabolites between TCE and chloral hydrate may be the cause of liver damage related to TCE, but it is not known if the generalized skin reaction was related. It would appear that CYPs may be involved in the pathogenesis of the manifestation of TCE toxicity.

TCE hypersensitivity syndrome is believed to be an idiosyncratic generalized skin disorder complicated by hepatitis, which resembles severe drug hypersensitivities. There is controversy as

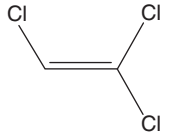

FIGURE 20.1 Chemical structure of trichloroethylene.

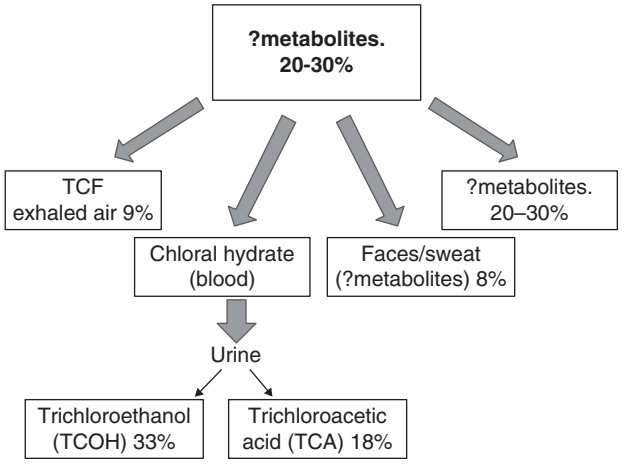

FIGURE 20.2 Metabolic pathways of trichloroethylene.

to whether the solvent itself or its impurities or stabilizers are the cause for the hypersensitivity reactions. Kamijima et al. reported a study that characterized the exposure of 19 hospitalized patients and their healthy colleagues by measuring TCE metabolites in their urine. Onsite surveys were conducted in six factories where the disorders occurred and in two control factories without such occurrences despite TCE use to assess the exposure of "healthy" workers. Urinalysis of the patients detected TCA in all of them. Its average concentration in the end-of-shift urine was estimated to be 206 mg/L. Onsite survey of healthy exposed workers revealed that the maximum urinary TCA concentrations and the maximum time-weighted average concentrations of personal TCE exposure were 318–1617 mg/L and 164–2330 mg/m^3, respectively. There was no common impurity in TCE used in the factories. Their results suggested that TCE itself caused the skin hypersensitivity disorders, and that the disorders occurred in factories where TCE metabolites could be extensively accumulated, possibly due to long working hours (6).

Active extracellular matrix degradation and remodeling are involved in the skin hypersensitivity reaction induced by chemical exposure. Yang et al. compared the effects of in vitro exposure to trichloroethanol (TCOH) and TCA of a keratinocyte cell line (HaCaT) and reported that TCOH-modulated matrix metalloproteinases (MMPs) (as marker of sensitization). A dose–effect relationship between TCOH treatment and MMP-9 activity, mRNA, and protein expression levels was found in HaCaT cells. TCOH also induced upregulation of TIMP-1 mRNA and protein. No such effects in HaCaT cells treated with TCA indicating that TCOH might play an important role in TCE-induced skin hypersensitivity (7).

Studies have also indicated that genetics may play a role in TCE cutaneous reactions (8). Acetylation catalyzed by *N*-acetyltransferase (NAT) is the major route of conjugation reaction of many xenobiotics. Two genes (NAT1 and NAT2) that encode for acetylation have been sequenced. NAT2 is reported to be involved in the glutathione-mediated metabolism of TCE. Recently, the relationship between TCE-induced generalized skin reaction and genetic polymorphisms of NAT2 were studied. NAT2 slow genotype significantly increased the risk of TCE skin reactions. Hence genetic polymorphism for NAT2 may be a factor that increases susceptibility to TCE-induced skin reactions (9). The toxic effects of TCE can be classified into acute and chronic toxic effects.

Watanabe studied two patients with TCE hypersensitivity syndrome and indicated that oxidative metabolites of TCE, which might include trichloroacetylated-protein adducts, could induce a generalized skin eruption. He identified human leukocyte antigen (HLA)-B*1301 and HLA-B*44 as markers of individual susceptibility to TCE-induced hypersensitivity syndrome (HS). Polymorphism of aldehyde dehydrogenase (ALDH), the major enzyme in TCE metabolism, appeared to be associated with TCE-induced HS. Watanabe concluded that this disorder is quite similar to drug-induced hypersensitivity syndrome (DIHS), also referred to as drug rash with eosinophilia and systemic symptoms (DRESS), from the perspective of the onset of the reaction after exposure to TCE/drugs, clinical manifestations, blood examination, and period of virus reactivation (10).

Acute Toxic Effects of TCE

1. Mucous membranes irritation affecting the eye, nose, throat, and respiratory tract.
2. Central nervous system toxicity where massive exposure can cause excitation, dizziness, and euphoria initially. These symptoms are often followed by headache, nausea, sleepiness, and in severe cases, coma.
3. Respiratory system inflammation due to chemical pneumonitis and in more severe cases respiratory failure and death.
4. Cardiac arrhythmias and cardiac failure due to TCE effects on the myocardium.

Chronic Toxic Effects of TCE

1. Skin: Common presentation includes irritant contact dermatitis, toxic erythema presenting as erythroderma and scaliness, and in severe cases generalized exfoliative dermatitis (GED). Other chronic cutaneous manifestations of systemic TCE toxicity include scleroderma (11), erythema multiforme-like eruptions, and Steven–Johnson syndrome (SJS) (12), subcorneal pustular eruption, and GED (14). The occurrence of such skin reactions often do not parallel to the level/amount of TCE exposure, that is, without significant dose–effect relationship. It is believed that these chronic skin manifestations of TCE toxicity are caused by inhalation and direct skin exposure/absorption.
2. Central nervous system: Chronic nonspecific complaints, such as headache, irritability, fatigue, and insomnia, may occur. Impaired psychomotor and behavioral tests have been observed. Alcohol intolerance characterized by cutaneous vasodilatation, especially in the face, has been reported.
3. Liver: Few cases of hepatitis-like syndromes and steatosis (fatty liver) have been reported to be associated with chronic TCE exposure.
4. Kidney: Altered renal function, for example, proteinuria and raised blood urea may occur.

TCE DERMATOTOXICOLOGY

Acute and chronic skin reactions to TCE through skin exposure and inhalation are well described in the medical literature.

TCE appears to be a strong skin sensitizer, whereas its metabolite, TCA, a moderate one. The other metabolites, namely, trichloroethanol and chloral hydrate, appear to be weak skin sensitizers. The cutaneous manifestations of TCE toxicity is believed to be mediated by an immunologic process. It has been suggested that the reaction may be a manifestation of a type IV allergic reaction with TCA as the allergen. This conclusion was based on the contact allergenic potential of TCE and three of its metabolites, namely, TCA, trichloroethanol, and chloral hydrate, based on a modified guinea pig maximization test (GPMT) (15). The study reported that the skin sensitization rate of TCE, TCA, and 2,4-dinitrochlorobenzene was 71.4%, 58.3%, and 100.0%, respectively, and no sensitization was observed from trichloroethanol and chloral hydrate. The histopathologic changes also appear to support that an allergic type reaction based on studies on the effect of TCE and TCA on guinea pig skin.

Goon et al. in their case report suggested that the skin reaction from TCE exposure could represent a hypersensitive reaction and preferred the term TCE hypersensitivity syndrome presenting with exfoliative dermatitis, mucous membrane erosions, eosinophilia, and hepatitis following exposure to TCE. In their patient who died from his illness, his urinary TCA level was not excessive. Whereas his work colleagues working near him were found to have higher urinary TCA levels, but none of them had any rashes or systemic complaints. Hence the authors concluded that, it may be that their patient did not have a toxic reaction to TCE but that he was hypersensitive to it, the difference being that a toxic reaction is predictable and will occur in most individuals at a given threshold dose, whereas hypersensitivity will occur only in a genetically predisposed individual, is idiosyncratic, and may occur at a very low dose. Goon et al. concluded that the term "TCE hypersensitivity syndrome" is probably more appropriate than SJS, as it highlights the extensive systemic involvement particularly of the liver, as well as the mucocutaneous involvement. The condition appears to have a potentially fatal outcome and is more severe than SJS (16).

Watanabe studied two patients with TCE hypersensitivity syndrome indicated that oxidative metabolites of TCE, which might include trichloroacetylated-protein adducts, could induce a generalized skin eruption. Watanabe concluded that this disorder is quite similar to DIHS, also referred to as DRESS, from the perspective of the onset of the reaction after exposure to TCE/drugs, clinical manifestations, blood examination, and period of virus reactivation (10).

EPIDEMIOLOGY OF TCE HYPERSENSITIVITY SYNDROME

There are relatively few reports of TCE dermatotoxicity in the medical literature. Most reports of TCE dermatotoxicity consist of single case report or small case series. Most cases arise from occupational exposure to TCE.

The number of patients suffering from TCE-related severe skin disorders with liver dysfunction has been increasing in developing countries in Asia. Most recent reports come from China. In Japan, five cases of this disease have been reported recently (10).

Bauer and Robens were that first to report cases of TCE dermatotoxicity. Four patients developed generalized dermatitis after exposure to TCE. One patient had hepatitis with transaminitis and hyerpbilirubinemia(17).

In another report three men showed an unusual toxic manifestation including toxic encephalopathy, hepatitis, and carpal spasm occurred among young, healthy workers, following exposure to TCE while carrying out degreasing procedures in the jewelry industry (18).

In a case series report, five patients were described to have developed SJS due to occupational exposure to TCE in Singapore. All five patients had an abrupt onset of fever, generalized erythema, maculopapular skin eruptions that later exfoliated, and conjunctivitis with typical features of SJS. All patients had hepatitis (with transaminitis) and hepatomegaly. Four recovered and one died of septicemia and liver failure (12).

In a case report from Japan, a 21-year-old printer developed exfoliative dermatitis with mucous membrane involvement, fever, and liver dysfunction after a two-week occupational exposure to TCE. The patient had positive patch test reactions to TCE and trichloroethanol, the metabolites of TCE. This dermatitis was considered to be mediated by a delayed-type hypersensitivity mechanism (19).

Schattner and Malnick reported a 48-year-old woman who presented with anorexia, vomiting, and fever. She developed hepatitis and uveitis following occupational exposure to TCE, used as a solvent in the factory where she worked for several years. Her job included cleaning parts with TCE in a cold bath for about 3 hours per day. Air samples for TCE was 8-fold the permissible exposure level. Six months after her illness commenced and when complete symptomatic and biochemical recovery was established, a rechallenge was made by the patient's return to her previous work. Within two weeks lassitude reappeared as well as an isolated significant increase in serum alkaline phosphatase. At that time air sampling for TCE revealed exposure of 550 ppm and TCA was discovered in the patient's urine (20).

Bond reported a 30-year-old man degreaser who experienced symptoms of weakness, dizziness, decreased appetite, nausea, abdominal pain, diarrhea, fever, chills, dry skin, red rash, peeling face, and itching one month after exposure to TCE. He had marked liver enzyme elevation without evidence of cholestasis. Tests for Hepatitis A, B, and C, CMV, and HIV1 were all negative. The night following his first day back at work he had a recurrence of a red, diffuse rash. Physical examination one week after re-exposure showed diffuse, erythematous rash; some peeling skin and pitting edema to the knees. Alanine aminotransferase (ALT) was raised. White blood cell count was 10,100/mm^3 with 27% eosinophilia. The levels of TCE or its metabolites in the patient's blood or urine were not reported. The patient was diagnosed to have sensitization to TCE, or its metabolites (21).

Chittasobhaktra reported a case of a young female who developed fever, erythema, and jaundice following occupational exposure to TCE. Liver biopsy showed liver cell necrosis in the centrilobular zone with polymorphonuclear leukocytes and lymphocytic infiltration in the portal vessels with multinucleated giant cells. A patch test showed positive reaction to 50% TCE (3).

Goon et al. reported a case of a 36-year-old Chinese man presented with fever, generalized erythematous maculopapular rash, conjunctivitis, and mucositis. Skin biopsy showed features consistent with erythema multiforme. He had a history of occupational exposure to TCE. The patient was diagnosed to have hypersensitivity to TCE. His clinical condition deteriorated rapidly and died after several days. The factory had been measuring air levels of TCE every six months. The levels had always been less than half

the threshold limit value of 50 ppm. Workers who were exposed to TCE in the factory have undergone an annual measurement of urinary TCA levels and their values were consistently below the biological threshold limit value of 100 mg/L. The urinary TCA levels of two of the three workers who had worked near the patient were found to be above the biological threshold limit value (100 mg/L). The levels were 148.2, 122.0, and 80.1 mg/L. However, none of them had any rashes or systemic complaints. The patient's urinary TCA level was not excessive (16).

TCE-induced hypersensitivity dermatitis is one of the serious occupational health events in China. The largest series of TCE dermatotoxicity reported so far comes from China (22). The authors investigated retrospectively 50 patients with dermatitis from TCE between 1997 and 2000. They reported that the occurrence of dermatitis did not parallel TCE exposure levels and without significant dose–effect relationship. The clinical manifestations included skin lesions, fever, superficial lymphadenopathy, and liver dysfunction. Infection was the major complication. The major clinical types of TCE dermatitis included exfoliative dermatitis and erythema multiforme. The authors suggested that the dermatitis is mediated by delayed-type (IV) hypersensitivity.

Xu et al. sampled and tested some cleaning agents from the companies where TCE-induced skin disorder occurred and measured TCE concentrations in the workplace air. The symptoms, signs, and laboratory test results of patients were collated. TCE concentrations varied from 10.2% to 91.4% in the cleaning agent TCE levels in the workplace air ranged between 18 mg/m^3 and 683 mg/m^3, at most sampled sites TCE levels were higher than China national health standard for TCE. The TCE exposure time of the patients was 5–90 days (average 38.2 days). The prevalence of patients with headache, dizziness, skin itch, and fever were 90.5%, 100%, 100%, and 61.9%, respectively; 85.7% patients had skin erythema, 90.5% with rashes, and 38.1% with blisters. In addition, liver enlargement occurred in three patients, the abnormal rate of ALT, aspartate aminotransferase, and total bilirubin were 90.5%, 85.7%, and 76.2%, respectively. Six out of 15 patients were with abnormal electrocardiogram, and TCA elevated in 14 patients (66.7%). The major detrimental effect of TCE was to induce hypersensitivity dermatitis and liver dysfunction, the occurrence of this disorder is probably related to the individual hypersensitivity to TCE exposure (23).

CLINICAL MANIFESTATION OF TCE HYPERSENSITIVITY SYNDROME

It is likely that the cutaneous and systemic manifestation, in particular hepatitis from TCE exposure reported above represents a unique hypersensitivity reaction on the skin and liver. Some authors have suggested that it is mediated through a type IV hypersensitivity reaction, which can be confirmed with patch testing with TCE or its metabolites. But the lack of association of TCE exposure levels and clinical disease and inconsistent patch test reactions in most reports would appear to suggest that an idiopathic hypersensitivity reaction is in play. Genetic susceptibility probably contributed to the manifestation of the disease.

Most cases of TCE hypersensitivity syndrome occurred within one month but can range from 5 to 66 days after the onset of exposure to TCE. The syndrome presents with skin eruptions (erythema, exfoliation, and erythema multiforme-like), fever, superficial lymphadenopathy, and liver dysfunction (with jaundice and hepatomegaly or hepatosplenomegaly). Most of the patients had no history of other causes of hepatitis, for example, drug abuse or herpes infection. The level of exposure to TCE of reported cases ranged from <9 to 800 ppm. In severe cases, the lesions involved mucous membranes including the conjunctiva and buccal and genital mucosae, and often the patients were diagnosed with SJS. Skin biopsy generally showed features consistent with erythema multiforme.

TREATMENT AND PROGNOSIS OF TCE HYPERSENSITIVITY SYNDROME

The key factors to treat this disease successfully included the use of timely administration of systemic glucocorticoid, professional skin care, active treatment to protect the liver, and to avoid infection(22,24).

From earlier reports it would appear that TCE hypersensitivity syndrome is often associated with extensive systemic involvement particularly of the liver, as well as mucocutaneous involvement. It is associated with severe morbidity and mortality. It seemed more severe than SJS.

Early identification of the syndrome is essential to reduce morbidity and mortality. Patients with exfoliative dermatitis, erythema multiforme-like skin eruptions with hepatitis should have a history of TCE exposure taken. If TCE exposure is recorded, patients should be removed from further exposure to TCE to reduce mortality risk.

The best prevention against TCE hypersensitivity syndrome remains controlling workers' exposure to TCE. Air level of TCE at workplace should be kept below the maximum allowable level. Workers exposed to TCE should have regular urine level of its metabolites checked as an indication of exposure level.

CONCLUSION

Toxicity to industrial solvents is a well-recognized occupational problem. Effective preventive measures can be introduced into the workplace. However, new and better industrial solvents will continue to be introduced into the workplace and new hazards from solvents will continue to be a hazard to workers. Cutaneous and systemic toxicity from TCE is well recognized.

TCE dermatotoxicity is often associated with systemic effect on the liver (hepatitis) presents with erythroderma, exfoliation, erythema multiforme-like skin lesions, mucositis (conjunctiva, oral, and genital) culminating with features of SJS with mortality.

Physicians managing patients with erythema multiforme-like skin eruptions and SJS should be alerted on TCE exposure as a possible causative factor.

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21 Chemical agents that cause depigmentation

Barbara Noury, Sahar Sohrabian, and Howard I. Maibach

INTRODUCTION

Many disorders result in disturbances of pigment formation by the melanocytes (1). Hypomelanosis or a decrease in the formation of the pigment melanin may be caused by many disorders. Leukoderma, derived from the Greek terms, λευκο white + δερμα skin, due to chemical exposure has been associated with several classes of compounds; most being phenols or thiols. These chemicals are useful as antioxidants and find utility in rubbers and plastics, in foods, and as polymerization inhibitors in monomers. Because of the widespread use of these chemicals, it is important to examine the effects of exposure and the mechanism of depigmentation.

HISTORY

Occupational leukoderma due to exposure to chemicals was first reported more than 65 years ago (2). The depigmentation, which may resemble vitiligo, was produced by the monobenzyl ether of hydroquinone (MBEH), which translocated from rubber gloves worn by workers. Once it was documented that chemical agents could depigment the skin, it became important to test them for this property, and several laboratory procedures have been developed for this purpose.

The methods used for testing depigmenting chemicals have been reviewed previously (3).

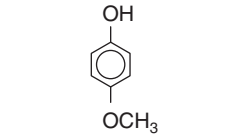
CHEMICAL STRUCTURES CAUSING DEPIGMENTATION

Chemical depigmentation has been associated with a variety of compounds (Fig. 21.1). Most are phenols (aromatic or aliphatic) or sulfhydryl compounds, but divalent metals that bind to melanin have also been implicated. These materials are useful as antioxidants and inhibitors of polymerization. Because of these properties, they are employed in a variety of products and can potentially contact many people during manufacture and use. In addition, some of these agents have been applied intentionally for the purpose of lightening hyperpigmented skin. The structures and acronyms of these materials are shown in Figure 21.1, but catechol (CAT) and phenol have not been included. MBEH has been used to intentionally depigment hyperpigmented skin in humans (4,5). The results were not satisfactory because the response had wide individual variation. Furthermore, depigmentation occurred at sites remote from the site of application. There was no depigmentation without some evidence of inflammation. In another study, MBEH was used at 10–33% concentration in lotions and ointments, and was deemed to give satisfactory results when used to treat hyperpigmentation in patients (6). Bleaching creams containing hydroquinone (HY) have also been reported to cause leukoderma (7). The mechanism is tyrosinanse inhibition, depletion of glutathione, and generation of reactive oxygen species.

Clinical data has been gathered from exposures to products containing depigmenting chemicals. Some ceramic lacquers contain phenolic compounds (exact structure unidentified), and one case of leukoderma has been reported from exposure to these materials (8). Leukoderma from contact with neoprene swim goggles has been reported (9), but the agent responsible was not identified. Hypopigmentation due to contact with phototypesetting paper containing *tert*-butyl catechol (TBC) has been described (10). TBC is also used as an antioxidant in industrial lubricants, and workers who come in contact with these experience depigmentation (11). Antioxidants are added to polyethylene film, and these materials can translocate if the film is in contact with skin. Polyethylene film, used as an occlusive dressing during steroid treatment produced two cases of leukoderma (12). Depigmentation due to adhesive tape was described (13), but the component in the tape was not identified because the subject refused to be tested with the individual components.

Phenols are a common ingredient in germicidal disinfectants. A study describing five cases of depigmentation in one hospital and seven in another was reported (14). The antiseptic used for cleaning surfaces in the hospital contained 4.1% of *o*-benzyl-*p*-chlorophenol (BCP) and 3% 4-tert-butylphenol (TBP). In addition, experimental studies were carried out on five volunteers who were tested with 6% TBP in 70% ethyl alcohol applied to the upper arm under occlusion. Maximal pigment loss occurred at approximately one month, and pigment returned in all subjects about one month later. Another group of subjects were tested with 6% hexachlorophene (HEX), *o*-phenyl phenol (OPP), BCP, and MBEH, and 1% solutions of *tert*-amyl phenol (TAP) and BCP. Depigmentation was produced in some subjects by all materials with the exception of MBEH. MBEH is capable of producing depigmentation as shown by other studies where a 20% solution was used (4,5).

Exposure to depigmenting agents can and does occur if proper handling procedures are not practiced during the manufacture. Thirteen cases of leukoderma have been described among workers in a plant producing OPP and *p*-phenyl phenol (PPP) (15). Two cases of leukoderma in a plant producing the monomethyl ether of hydroquinone or 4-hydroxyanisole (HA) were described, although 169 other men in the same plant were examined and showed no sign of depigmentation (16). HA is used as a stabilizer of vinylidene chloride, and two cases of leukoderma have been described in a plant where the material was being made (17). A plant making TBP had 54 of 198 men with leukoderma; the intensity of the disorder was related to the degree of exposure (18). Nine cases of leukoderma was seen in two plants engaged in the production of TBP, butylated hydroxytolutene (BHT), and TBG (19).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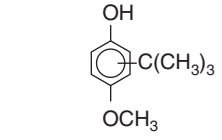
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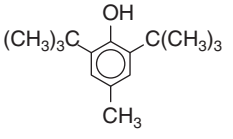
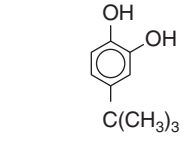

Butylated hydroxy toluene (BHT)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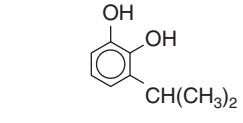
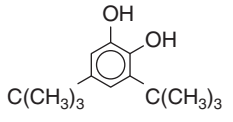
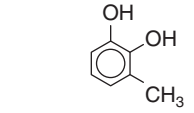
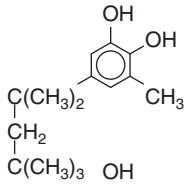
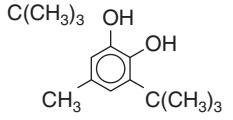

3-Isopropyl catechol (3IC)


3,5-Diisopropyl catechol (DIC)


3-Methyl catechol (3MC)


3-Methyl 5-*tert.* octyl catechol (MOC)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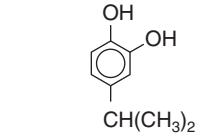
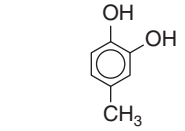
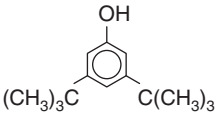
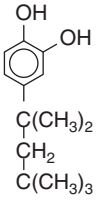
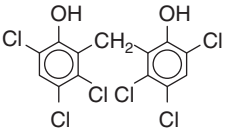
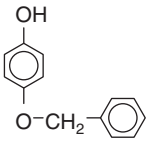

4-Isopropyl catechol (4IC)

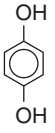

4-Methyl catechol (4MC)

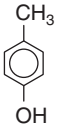

3,5-Ditertiary butyl catechol (DTBC)

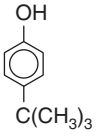

4-*tert.* Octyl catechol (4OC)

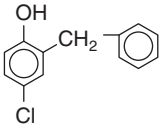

Hexachlorophene (HEX)


Hydroquinone monobenzyl ether (MBEH)

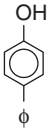

Hydroquinone (HY)


p-Cresol (CRE)


4-*tert.* Butyl phenol (TBP)


o-Benzyl-*p*-chlorophenol (BCP)


o-Phenylphenol (OPP)


p-Phenylphenol (PPP)

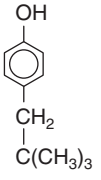

p-tert. Amylphenol (TAP)

FIGURE 21.1 Compounds that have caused depigmentation.

In addition to the clinical observations, experimental studies have identified many compounds that cause hypomelanosis. Laundry ink containing p-cresol produced depigmentation in CBA/J mice (20). Thirty-three compounds were tested in black guinea pigs (21). Of these, 12 compounds produced depigmentation to some degree. Those that were strong depigmenters were TBC, 4-isopropyl catechol (4IC), 4-methyl catechol (4MC), and CAT. Some produced definite but moderate hypopigmentation, among them, 3-isopropyl catechol (3IC), 3,5,-diisopropyl catechol (DIC), HY, 3-methyl catechol (3MC), and 3-methyl-5-*tert*-octyl catechol (MOC). Others produced definite but weak depigmentation: 3-*tert*-butyl 5-methyl catechol (BMC), 3,5-ditertiary butyl catechol (DTBC), and 4-*tert*-octyl catechol (4OC). Twenty-two additional compounds are listed in Table 21.1; some produced depigmentation and others did not. Substitution in the 4 position confers greater activity than the same substituent in the 3 position; for example, 4-methyl catechol is more potent than 3-methyl catechol. Some, but not all, compounds containing a sulfhydryl group are capable of producing depigmentation. β-Mercaptoethylamine hydrochloride (MEA) and *N*-(2-mercaptoethyl)-dimethylamine hydrochloride (MEDA) were strong depigmenting agents. 3-Mercaptopropyl amine hydrochloride and cystamine hydrochloride are weak-to-moderate depigmenters. Sulfanilic acid, cystamine, *bis*-(2-amino-1-propyl)disulfide, 2-(*N*,*N*-dimethylamine) ethanethiol S-acetate, 2-mercaptopropylamine hydrochloride, and α-mercaptoacetamide were weak depigmenters. Another study compared HQ, MEA, and MEDA in black guinea pigs (22). There is not as clear a pattern of structure–activity relationship among the thiols as there is with the phenols.

Another study on 23 compounds was carried out in black guinea pigs and black mice (23). Strong depigmentation was found with HA, TBG, TAP, and MEBH. Moderate depigmentation was noted with HQ, TBP, phenol, and CAT. They failed to find depigmenting properties when testing butylated hydroxyanisole (BHA), BHT, octyl and propyl gallate, ethoxyquin gum guaiac, diethyl amine, hydrochloride, dilauryl thiodiproprionate, nonyl phenol, *o*-phenyl phenol, *p*-phenyl phenol, octyl phenol, nordihydroguaiaretic acid, and tocopherol. All the compounds mentioned last are used in a variety of products with which a large population comes in contact.

Condom leukoderma was reported in India (24), the patient tested positive with mercaptobenzothiazole and condom latex. The patient was treated with UVB therapy with repigmentation in eight weeks.

Cases of depigmentation induced by herbal oils were described after use of *Sesamum indicum* oil. This oil is used as an emollient (25).

Fatty acids, such as linoleic, linolenic, and oleic acid, may decrease of the amount of active tyrosinase inside the melanocyte (26).

α-Tocopherol can be a depigmenting product because it blocks dopaquinone and subsequent chemical oxidations in the polymerization pathway leading to the pigment (26). Diphenylcyclopropenone used for alopecia has shown some hypopigmentation. Toxicity is due to phenolic structure (26).

Methimazole (1-methyl-2-mercaptoimidazole) is considered as a weak depigmenting agent as a result in a study conducted on Brown Guinea Pig skin by Kasraee (27).

Henna or temporary tattoo, which sometimes contains paraphenylenediamine, was reported to cause depigmentation after contact allergy in a young Australian girl—it took 9 months to normalize (28).

Poly vinyl chloride is combined with many plasticizers, such as phthalates, which can cause depigmentation. Two cases were reported, one with stethoscope earpiece (37) and one with nasal canula (29).

TABLE 21.1
Compounds Tested in Black Guinea Pigs

Compound	Depigmenting potency
1,2,4-Trihydroxybenzene	0 to ±
2-Hydroxy-l,4-n apthoquinone	0
2,3-Dihydroxybenzoic acid	0
Sulfanilic acid, pH 3.9	0
bis(2-Amino-1-propyl) disulfide	±
β-Mercaptoethylamine hydrochloride	± to + +
2-Aminoethanethiol *S*-acetate	0
2-Mercaptoisopropyl amine	0 to ±
Hydrochloride × mercaptoacetic acid	0
2-Ethyl-*n*-hexyl-diphenylmethylene cyanoacetate	0
2,3,5,6-Tetrahydroxyquinone	0
3,4-Dihydroxyphenylacetic acid	0
Sulfanilic acid, pH 7	0
bis(2-Aminoethyl) disulfide or cystamine	0 to ±
2-Hydroxypyridine	0
N-(2-mercaptoethyl)dimethylamine hydrochloride (MEDA)	± to + +
2-(*N*,*N*-dimethylamine) ethanethio-*S*-acetate	±
Cystamine hydrochloride	± to +
α-Mercaptoacetamide	0 to ±
1,3-Propane sultone	0
3-Hydroxypropane sodium sulfonate	0

Note: Criteria for assessing activity are as follows: 0, no visible depigmentation and skin color similar; ±, small spots or speckles of depigmentation; +, uniform hypopigmentation; + +, complete dipigmentation.
Source: From Ref. 21.

REPIGMENTATION

Repigmentation after exposure to depigmenting agents is highly variable. Aside from individual variation, it is related to the degree and length of exposure to the agent. After application of MBEH for 30 days, repigmentation occurred one month after cessation of application (6). After workers ceased wearing rubber gloves containing MBEH, repigmentation commenced but the degree of repigmentation was not stated (2). Black subjects tested with 20 or 5% MBEH (4) had one subject who depigmented in one month and repigmentation was complete two months later. Depigmentation resulting from rubber swim goggles containing a depigmenting agent gradually repigmented over an eight weeks period after use of the goggles was discontinued (9). MBEH was used to depigment black subjects, and in some, white patches remained after two years and the investigators speculated it might be permanent (5). Some subjects tested with TBP repigmented within six months but others remained depigmented after one year (14). Those areas that depigmented least, repigmented first.

MECHANISM OF ACTION

The biosynthesis of melanin is a complex process that involves several steps, several of which are still not known. For example, several different protein structures can condense with indole quinone or 5-*S*-cysteinyldopa to give different colored pigments. Some of this is under genetic control, and thus is different in individuals as well as species. This process is shown in Figure 21.2. Pigment formation can be disrupted by interference at any of these steps. Seven mechanisms have been suggested by which the chemical agents could be producing depigmentation (21):

1. The agent may act selectively on a specific cell. The phenols do structurally resemble some of the intermediates involved in the synthesis of melanin, such as tyrosine or DOPA. Menter (30) tested eight compounds as substrates for tyrosinase and found them all suitable. Among them were the depigmenting agents TBC, 4MC, HA, and BHT. The presence of dopa-melanin enhances the action of tyrosinase on these substrates. It has been suggested that some of these products may act as antimetabolites and lead to degeneration or death of the cell (31).
2. Chemical agents, such as 4IC, may react and tie up the active sites of tyrosinase. The molecule does not have the necessary side chain for conversion to the indole quinone and the reaction may bog down at step III (Figure 21.2).
3. The agent can inhibit the melanin formation by blocking the enzymatic oxidation of tyrosine to DOPA and the subsequent conversion to melanin.
4. The agent can interfere with the biosynthesis of the organelles—premelanosomes and melanosomes. Melanin is a free radical and produces a signal when analyzed by electron spin resonance. The addition of HA to the system changes and increases the signal (32). Free radicals are capable of generating peroxides and disrupting cell and organelle membranes by lipid peroxidation. Investigations by electron microscopy have shown disruption of melanosomes and destruction of membranous organelles in melanocytes (33).
5. The agent can interfere with the biosynthesis of the protein (e.g., by combining with the melanocytic ribosomes, which appear to be the sites for tyrosinase synthesis) (33). This may be another facet of lipid peroxidation.

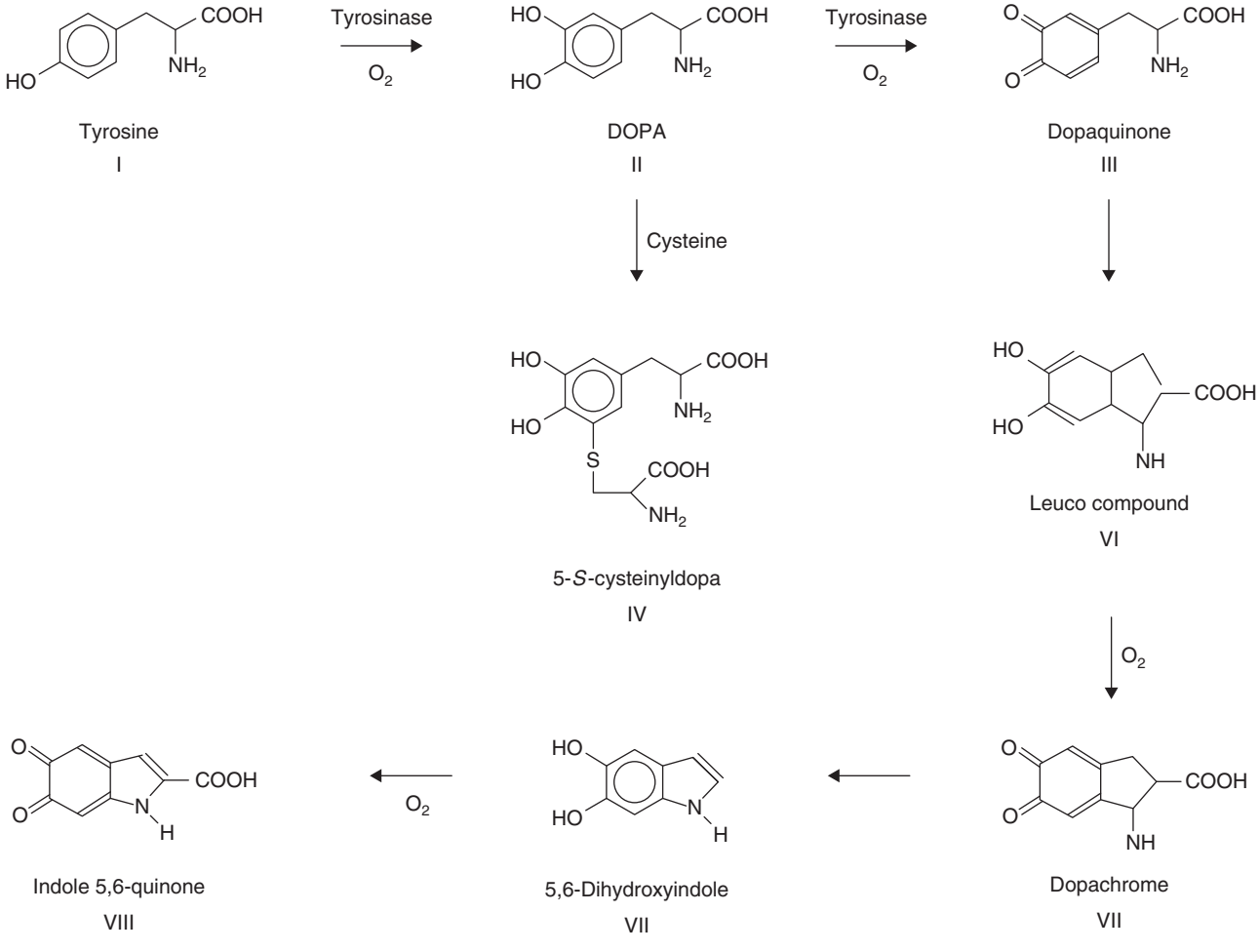


FIGURE 21.2 Steps in the synthesis of melanin (35). Indole 5,6-quinone undergoes condensation with proteins to form eumelanins, which are black or brown. The cysteine conjugate, 5-*S*-cysteinyldopa is further oxidized to a quinoid structure and then is conjugated with proteins to form pheomelanins, which are red or yellow in color. Tyrosinase activity can be diminished by substrate inhibition and, since several depigmenting agents with a phenolic structure can act as substrates, this may be one mechanism (30,36).

6. The agent can interfere with the transfer of melanosomes to keratinocytes, either by inhibiting the arborization of melanocytic dendrites or by causing intercellular edema. Irritation plays a role in pigment loss (23,5). Irritation is accompanied by edema, thus this may be a factor in depigmentation.
7. The agent can chemically alter the melanin present in the melanosomes. Because of the reducing action of some of the thiols, it appears that the dark-colored, oxidized form of melanin could be altered to the lighter-colored, reduced form. Since cysteine can condense with DOPA to yield pheomelanins, it may be possible for other thiols to be involved in a similar reaction. It has also been suggested that depigmenting agents may act as an antigen after increased cellular permeability due to inflammation. An antibody is formed and this stops the formation of melanin granules. If antigen is produced in sufficient quantities, the reticuloendothelial system could respond (5). This hypothesis has not been tested but it may explain depigmentation at remote sites (11).

Another mechanism that alters the level of glutathione reductase, which in turn affects the level of the reduced form of glutathione, may involve a change in the type of pigment produced (38). Hairless mice treated with TBC showed an increase of the enzyme glutathione reductase. It was suggested that this change increases the level of reduced glutathione and in turn increases the number of pheomelanins, which are lighter in color than eumelanins.

Some important findings have been made using the cultures of human melanoma cells (34). In this study, intracellular glutathione was depleted by treating the cells with buthionine-*S*-sulfoximine (BSO). Tyrosine hydroxylase activity increased in parallel with glutathione depletion. The effect of thiols on melanogenesis can occur by at least two different mechanisms. First, low molecular weight thiol compounds can inhibit melanogenesis by direct interaction of the thiol groups with the tyrosinase active site, thus inhibiting tyrosine hydroxylation. Second, thiol groups are able to react with L-dopaquinone to form dopa-thiol conjugates that are pheomelanogenic precursors.

CONCLUSIONS

Many chemicals have been identified as depigmenting agents from clinical observations and experimental studies. These agents fall into primarily two categories: phenols and thiols. The most potent phenols are those containing an alkyl substitution in the 4 position. Those that are most irritating to the skin have the greatest potential for depigmentation. The mechanism by which depigmentation occurs is probably related to interference with one or more of the many steps of melanin biosynthesis. It is accompanied by the destruction of melanocytes and their organelles. The structure–activity relationship of the thiols is much less defined. The mechanism of action of the thiols may be related to the depletion of glutathione or the involvement of the thiol in the place of glutathione in the formation of melanin. Fortunately, experimental methods have been developed to test compounds for depigmenting properties. Once the potential is recognized, proper protective measures can usually be instituted to minimize human exposure. The human is more sensitive than other species, and there is a large variation in sensitivity among individuals.

Recent literature reviews (PubMed and Embase) fail to reveal new depigmenting agents and mechanisms. We question whether this represents lack of interest, few reportable cases, or that the depigmenters have been identified and removed from the environment. The authors welcome new information that may have escaped our notice.

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22 Hydroxychloroquine-induced retinopathy*

Aziza A. Wahby, Jackie M. Tripp, and Howard I. Maibach

ANTIMALARIALS IN DERMATOLOGY

Antimalarials have been used in dermatology for the management of cutaneous lupus erythematosus and connective tissue diseases (2). Other diseases treated with these agents include polymorphous light eruption, cutaneous sarcoidosis, and porphyria cutanea tarda. In the past, chloroquine was the primary antimalarial used in dermatology, and its adverse effects have been reviewed elsewhere (3). Currently, the antimalarial of first choice, at least in the treatment of cutaneous lupus, appears to be hydroxychloroquine (4). This drug tends to be viewed as the safest antimalarial, without any obvious difference in efficacy from other antimalarials (2).

One adverse effect of antimalarials, irreversible retinopathy, is characterized by a "bull's eye" depigmentation of the retinal pigment epithelium in the central macula. Hydroxychloroquine is generally regarded as safer than chloroquine (5), with a suggested incidence of chloroquine maculopathy between 1% and 6% and of hydroxychloroquine maculopathy below 1% (6). Hydroxychloroquine may be less retinotoxic than chloroquine because of its inability to cross the blood–retinal barrier (7), but there is a lack of conclusive data on the pathophysiology of antimalarial-induced irreversible retinopathy.

This rare but serious ocular effect of antimalarial therapy necessitates careful dosing and close ophthalmologic monitoring. Table 22.1 describes measures taken to minimize the retinal toxicity of hydroxychloroquine. However, despite these measures, patients still develop retinal disease from hydroxychloroquine. There is little evidence as to why this occurs in some patients but not in others, nor are there data to suggest a foolproof way of avoiding this complication.

OPHTHALMOLOGIC SCREENING

The frequency with which patients taking hydroxychloroquine are screened for retinal toxicity has been variable. According to the American College of Rheumatology (8), baseline ophthalmologic examinations are recommended, to be followed by subsequent examinations every 6–12 months. The Physician's Desk Reference (manufacturer's package insert) (9) recommends examinations every three months. Recommendations by dermatologists vary, but one approach (4) is to have patients examined at baseline and then every six months for the first year, followed by yearly evaluations. Although no definitive data exist, the American Academy of Ophthalmology (AAO) (10) notes that a screening regimen of this frequency does not appear to be cost-effective given the low incidence of this complication. The AAO defines low-risk patients as those receiving <6.5 mg/kg/day of hydroxychloroquine for <5 years and high-risk patients as those using the medication for long periods (>5 years, with a suggested fivefold increase in retinal toxicity after seven years (11)), those on larger doses (>6.5 mg/kg/day for short individuals), a cumulative dose >1000 g, those with kidney or liver disease, those with concomitant retinal disease, and those >60 years of age. Although these guidelines represent reasonable factors of which to be aware, there remains controversy as to a reliable index of patient characteristics accurately predicting the likelihood of retinopathy in hydroxychloroquine users. Recent analysis of the AAO's proposed risk factors by retrospective chart review (12) suggests that age and duration of therapy pose the greatest threat. Conversely, a study of 3995 patients with either rheumatoid arthritis or systemic lupus erythematosus proposed that age (along with weight and daily dose) is, in fact, unrelated to the incidence of toxicity (11). The AAO suggests that all patients undergo a baseline examination within the first year of treatment, after which high-risk patients should be seen at annual followup, whereas low-risk patients with a normal baseline examination do not require followup for the next five years. Patients and health care professionals must understand that, rarely, toxicity does occur earlier and at doses below the suggested threshold. The wide variety of suggested screening regimens described above prompted the need for this new consensus guideline from the AAO. The AAO recommendations attempt to ensure reasonable and effective ophthalmologic screening, while considering many issues including the rarity of this complication, the risk–benefit ratio of screening, and the potential for disease progression despite early detection with screening.

The only known treatment of antimalarial retinopathy is cessation of the medication, which unfortunately does not necessarily stop progression of the damage. Currently, in addition to appropriate dosing, early recognition is the best defense against serious vision loss (10). Table 22.2 lists the examinations employed by ophthalmologists in the evaluation of hydroxychloroquine retinal toxicity. Also included is a list of techniques regarded as insufficient for screening.

DOSING

Earlier dosing regimens for hydroxychloroquine emphasized the association between toxicity and daily ingestion exceeding 6.5 mg/kg of body weight. More current studies (10,11), however, report that most cases of retinotoxicity occur after seven years of

*Updated from Ref. 1, with permission.

TABLE 22.1
Dosing and Monitoring Measures for Minimizing Hydroxychloroquine-Induced Retinopathy

Measure	Comment/suggested approaches	References
Laboratory investigations	No current serologic testing can predict or detect retinal toxicity	(2,4)
Ophthalmologic evaluation	Baseline, then every 6–12 mo	(8)
	Baseline, then yearly followup for high-risk patients[a], no routine followup in low-risk patients for the first 5 yr	(9)
	Baseline then every 6 mo for the first year, followed by yearly evaluation	(10)
Dosage by bodyweight	Ideal bodyweight is used to calculate the maximal daily dose of 6.5 mg/kg/day	(4)
Evaluation of renal function	Dose may need to be decreased with diminished GFR; avoid administration in severe renal impairment (GFR <10 mL/min)	(2)
Evaluation of hepatic function	Antimalarials should be used with caution in patients with hepatic impairment. The exact effect that hepatic disease has on the development of retinal toxicity has yet to be described	(2,4)
Dosage in the elderly	Dose may need to be decreased in the elderly. Specific age-related dosage recommendations for antimalarials have yet to be reached	(33)

[a]Characteristics of high-risk patients include taking the medication for long periods (>5 years), taking higher doses (>6.5 mg/kg/day), cumulative dose exceeding 1000 g, kidney or liver disease, concomitant retinal disease, and age >60 years.
Abbreviation: GFR, glomerular filtration rate.

TABLE 22.2
Chloroquine and Hydroxychloroquine Screening Procedures (9)

Timeline	Baseline examination within first year of use
	Annual screening after 5 yr of use
Recommended screening procedures	
Ocular examination	Dilated retinal examinations are important for detection of associated retinal disorders, but should not be relied on for screening (low sensitivity)
Automated visual field	White 10-2 threshold testing. Interpret with a low threshold for abnormality, and retest if abnormalities appear.
In addition, if available, perform one or more of the following objective tests	
SD-OCT	Rapid test that can be done routinely; can show abnormalities very early, even before field loss
mfERG	Valuable for evaluation of suspicious or unreliable visual field loss; may show damage earlier than visual field testing
FAF	May validate other measures of toxicity; can show abnormalities earlier than field loss
Not recommended for screening	
Fundus photography	Recommended for documentation, especially at baseline, but now sensitive for screening
Time-domain OCT	Insufficient resolution for screening
Fluorescein angiography	Use only if corroboration of pigmentary changes is needed
Full-field ERG	Important for evaluation of established toxicity, but not for screening
Amsler grid	Use only as adjunct test
Color testing	Use only as adjunct test
EOG	Questionable sensitivity

Abbreviations: EOG, electro-oculogram; FAF, fundus autofluorescence; mfERG, multifocal electroretinogram; SD-OCT, spectral domain optical coherence tomography.

therapy, which coincides with reaching a cumulative dose of 1000 g at a typical daily dose of 400 mg, regardless of weight. Note that 400 mg/day falls below the 6.5 mg/kg/day cutoff except in short individuals.

With awareness of the increased risk of toxicity after 1000 g of hydroxychloroquine, regulating the total daily dosage administered remains crucial for minimizing the development of retinal disease. The currently recommended maximal daily dose for hydroxychloroquine is 6.5 mg/kg of ideal bodyweight (IBW), while for chloroquine it is 3 mg/kg of IBW per day (12). These recommendations are based on a retrospective analysis of >900 patients by Mackenzie (13). The eight patients with retinopathy associated with hydroxychloroquine received doses of between 5.59 and 9.30 mg/kg of actual bodyweight per day, but when IBW was used to calculate their true doses, these patients had been receiving 6.9–10.30 mg/kg. This study emphasized the importance of basing the milligram-per-kilogram dosage calculations on IBW rather than actual bodyweight. If the actual bodyweight of an obese patient was used to calculate the required dose, the lean tissues would be overdosed on a milligram-per-kilogram basis because fat, brain, and bone do not absorb much antimalarial medication. IBW (value in kilograms) is calculated using the following formulas (12):

IBW (males) = 50 + 2.3 for every inch above 5 feet
IBW (females) = 45.5 + 2.3 for every inch above 5 feet

In addition to accounting for body composition, renal and hepatic function must also be assessed. Since antimalarials are excreted through the kidneys and metabolized in the liver, renal and/or hepatic insufficiency may potentially contribute to toxicity (13).

TOXICITY DESPITE ADHERENCE TO DAILY DOSAGE GUIDELINES

A study (14) of 1207 patients taking hydroxychloroquine at doses of <6.5 mg/kg/day failed to show retinal toxicity. A 1991 analysis (15) of all published cases and reports of hydroxychloroquine-induced retinopathy found only two patients who developed true hydroxychloroquine-related retinopathy at doses of <6.5 mg/kg/day, and both of these patients had been taking the medication for >10 years. Since then, cases of retinal toxicity have continued to be reported in patients with normal renal function, despite dosages being kept below the recommended limits (16–22).

Weiner et al. (16) described a 60-year-old woman who developed retinopathy while taking hydroxychloroquine 6.1 mg/kg/day over a 20-year period. Falcone et al. (17) described a 70-year-old woman taking hydroxychloroquine 3.9 mg/kg/day for rheumatoid arthritis who developed maculopathy after seven years of therapy. This patient did not exceed 6.5 mg/kg/day even when IBW was used in the calculation. Mavrikakis et al. (18) reported two women (a 39-year-old with rheumatoid arthritis and a 58-year-old with lupus erythematosus) who developed retinal toxicity while taking hydroxychloroquine <6.5 mg/kg/day for 6.5 and 8 years, respectively. Bienfang et al. (19) reported two women (a 60- and a 75-year old) who developed retinal toxicity from hydroxychloroquine while taking 3.4 and 4 mg/kg/day for 15 and 5 years, respectively. Warner (20) discussed the case of a 45-year-old woman who was taking hydroxychloroquine 5.9 mg/kg of IBW per day and developed retinal toxicity after 7.5 years of therapy. Alarcon (21,22) presented a case of retinal toxicity developing in a 35-year-old woman who took hydroxychloroquine 6.25 mg/kg of IBW per day for 10 months. Unfortunately, in many of these reports, the patients' height was not disclosed and it is unclear if IBW was used in the dose calculation. These cases illustrate not only the importance of carefully monitoring daily and cumulative doses but also the difficulty in predicting which patients will develop toxicity.

INTERINDIVIDUAL DIFFERENCES

Reports of patients developing retinal toxicity despite receiving doses of hydroxychloroquine below the currently recommended maximum therapy suggest the possibility that interindividual differences may have played a role.

The mechanism of retinal toxicity is unclear, but from the reported cases of patients taking "safe" doses, it seems that the condition is slow to develop. This makes sense, as hydroxychloroquine, given at 200 mg/kg/day, achieves a steady state at approximately 6 months (23). One dose–response study (24) suggested that steady-state levels of hydroxychloroquine could be reached at about six weeks by giving doses of >400 mg/kg/day, thereby providing an argument for use of dose loading to achieve steady state sooner. The same study (24) showed that there was no statistically significant dose–response relationship for ophthalmologic adverse events. The ophthalmologic events that were observed included macular, color vision, visual acuity, and foveal reflex abnormalities. Retinal toxicity was not observed, probably because of its rarity and the limited length of the study (six months). There was no evidence that the ocular abnormalities observed in this study had any bearing on hydroxychloroquine-induced retinopathy, and because of its evolution over long periods of therapy, the applicability of such short-term dosing data is questionable.

Because pharmacokinetic factors influence the concentrations achieved with a specific dosing regimen for many drugs, examining the pharmacokinetic properties of hydroxychloroquine would be an ideal starting point for addressing interindividual differences as well as toxicity parameters.

Concentrations of hydroxychloroquine are usually measured in the blood rather than in the plasma, as whole blood assays allow for greater sensitivity and are technically easier to conduct (25). A commonly employed method of measuring blood concentrations of hydroxychloroquine is high-performance liquid chromatography (HPLC), which is described in detail by Tett et al. (26).

Although most reports of retinal toxicity occur after years of treatment, some data from existing pharmacokinetic studies that have examined treatment over a relatively shorter time frame may shed some light on interindividual differences. One study (27) prospectively measured blood concentration differences in 23 patients taking hydroxychloroquine at doses of either 200 or 400 mg/day over the course of six months. The mean hydroxychloroquine blood concentrations at six months were significantly different between the two groups, indicating a dose–concentration relationship. However, even though there was a significant difference between blood concentrations in the high-dose group and those in the low-dose group, there was a wide variability in hydroxychloroquine blood concentration between patients, with overlap of concentrations between the groups. Those receiving the lower dose had blood concentrations of hydroxychloroquine at six months that varied from 69.4 to 996.7 ng/mL, and those receiving the higher dose had concentrations ranging from 370.8 to 1574.3 ng/mL. The variability in blood concentrations noted in subjects taking comparable doses may have been due to an inherent variability in the HPLC detection technique. However, the authors hypothesized that the disparate blood concentrations seemed to reflect significant interindividual differences in oral absorption and clearance of hydroxychloroquine. These differences could potentially impact upon the approach to minimizing retinal toxicity.

As no obvious relationship between hydroxychloroquine concentrations and ocular adverse effects was observed, the relationship between toxicity and hydroxychloroquine metabolites was examined (28). One hundred twenty-three patients without renal or hepatic disease received a loading dose of hydroxychloroquine varying from 400 to 1200 mg/day, followed by treatment with 400 mg/day for a total of 24 weeks of therapy. Hydroxychloroquine is metabolized oxidatively to desethylhydroxychloroquine, desethylchloroquine, and bisdesethylchloroquine, and blood concentrations of each of these chemicals were measured by HPLC. Although not statistically significant, there was a trend toward higher blood bisdesethylchloroquine concentrations in patients reporting eye problems compared with those not reporting ocular adverse events. Specific problems that were observed included visual field, color vision, Amsler grid, and foveal reflex abnormalities. Not surprisingly (again because of its rare occurrence) there was no evidence of maculopathy. Furthermore, none of the ocular problems observed was specifically related to retinopathy. So, although bisdesethylchloroquine may have had some relationship to ocular toxicity, there is no evidence that it had any relationship to hydroxychloroquine retinopathy.

A pharmacokinetic model for hydroxychloroquine has recently been developed (29). It is theorized that by using this model to achieve targeted concentrations, between-subject variability can be decreased. However, neither this model, nor any other in the literature, considers in detail the dosage of hydroxychloroquine based on ideal or lean bodyweight, despite the widely accepted recommendation (13) to dose hydroxychloroquine in such a manner. It is therefore unclear what effect, if any, this model will have on solving the issue of hydroxychloroquine-induced retinal toxicity.

Lastly, there has been some suggestion that interindividual variability in the retinal response to hydroxychloroquine may be genetic. Shroyer et al. (30) identified a photoreceptor-specific adenosine triphosphate-binding cassette transporter gene, ABCR (also known as ABCA4), which was mutated in Stargardt disease. Because this condition shares phenotypic similarities with antimalarial retinopathy, they examined eight patients with retinopathy from chloroquine or hydroxychloroquine for this gene. Two of the eight subjects had ABCR mutations and another three had missense changes that were different from a large group of controls. The significance of these findings is unclear: the ABCR mutations may be coincidental findings in patients with antimalarial retinopathy, or the mutations may be linked to the patients' retinopathy independent of antimalarial exposure. Nonetheless, the idea that antimalarial exposure in the face of pre-existing genetic susceptibility can lead to retinal degeneration is interesting.

CONCLUSIONS

Irreversible retinopathy is a rare but potentially serious adverse effect of hydroxychloroquine. One approach to lifting this effect is to arrive at a proper dosage strategy. The current strategy is based on limiting the daily dose of hydroxychloroquine to <6.5 mg/kg/day, with the weight in kilograms being IBW. This approach is complicated by the fact that there are no good pharmacokinetic models for hydroxychloroquine with regard to ocular toxicity and thus no objective validation that using IBW is indeed the best method. Indeed, as evidenced by the case reports discussed above, the idea of basing hydroxychloroquine dosage on a milligram-per-kilogram limit is a good, but not absolute, rule of thumb. Clearly, despite current dose limitations, retinal toxicity with hydroxychloroquine is still occurring, albeit rarely.

In addition to not exceeding maximal dosages, ophthalmologic monitoring is extremely important for patients taking hydroxychloroquine. Although extensive controversy exists with regard to optimal frequency of ophthalmologic followup (2,4,7,8), it clearly has a role in limiting further retinal damage. The most recent guidelines issued by the AAO (10) seem reasonable and appear to consider the practical nature of screening for such a rare but serious complication. However, while having a patient taking hydroxychloroquine monitored by an ophthalmologist is necessary, it is not an absolute preventative method.

The significant overlap of blood concentrations of hydroxychloroquine among patients receiving a wide range of doses suggests that prevention of retinal toxicity may occur by achieving specific drug concentration targets. The assessment of this hypothesis cannot proceed without an accurate pharmacokinetic model that examines hydroxychloroquine-induced retinal toxicity and takes into account such measurements as ideal or lean bodyweight. Developing this model is crucial to evaluating the nature of hydroxychloroquine toxicity in relation to bodyweight and other potential factors.

The utilization of modern computational chemistry techniques, such as quantitative structure–activity relationship (QSAR), may prove valuable in determining the pathophysiology of antimalarial retinotoxicity, perhaps paving the way for hydroxychloroquine variants with increased efficacy and decreased toxicity. Exploration of the enzyme glycogen synthase kinase-3β's (GSK-3β) structure and subsequent generation of a QSAR equation suggested that it had at least two distinct binding sites available for pharmacologic inhibitors, one of which was hydroxychloroquine (31). GSK-3β is a mammalian kinase with three homologs on the *Plasmodium falciparum* genome (32). In malaria, *P. falciparum* transfers this enzyme into host erythrocytes, where it is believed to regulate the parasite's circadian rhythm. Although the sensitivity of *Pf*GSK-3 to pharmacologic inhibitors is not identical to that of human GSK-3, further investigation could elucidate hydroxychloroquine's mechanism of action in rheumatic diseases and maculopathy. Analogous studies utilizing QSAR might reveal portions of hydroxychloroquine's pharmacophoric space subject to alterations that could decrease its retinal toxicity and increase its effectiveness in clinical practice. This type of analysis is likely precluded by our limited understanding of the pathophysiology of retinal disease.

Taken together, in spite of half a century of antimalarial use, much remains to be resolved. Adherence to current maximal drug dosage recommendations and careful followup of treated patients remain indicated until vital questions regarding hydroxychloroquine-induced retinopathy are answered.

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23 Factors influencing applied amounts of topical preparations

Nikolay V. Matveev, Tanzima Islam, and Howard I. Maibach

The effect of any medication depends on the applied dose. In case of topical medications, the applied amount is often uncertain and variable. The importance of proper dosage of topical medications in dermatology was seldom discussed, but it was demonstrated that accurate dosage of topical agents (e.g., calcipotriol) helped to provide better efficacy (1). Additionally, to make any conclusion regarding the effectiveness of a topical preparation in clinical trials, it is preferable to have the amount of a topical preparation known and well controlled.

Unfortunately, only limited scientific data are available on the variability of the amount of topical preparations applied to the skin in different conditions. Some studies concerned not pharmaceutic preparations, but sunscreens; others dealt with application of barrier creams. Nevertheless, with certain limitations, the data of sunscreen and barrier cream studies might be generalized to all topical preparations, including topical pharmaceutics.

Our aim was to collect and analyze the available information on the factors influencing the amount of various topical agents applied to skin.

The analysis of the data demonstrated that the amount of applied topical agents depended on several factors, which can be divided into two main groups: (*i*) preparation dependent and (*ii*) patient dependent.

PREPARATION-DEPENDENT FACTORS

Preparation-dependent factors are (*i*) form of a preparation (e.g., ointment, cream, lotion); (*ii*) the physical characteristics of the preparation (e.g., its viscosity); and (*iii*) type of container.

The Form of Preparation

When fixed amounts of ointment, cream, or solution are distributed on the skin of volunteers, it was demonstrated that the ointment was most evenly distributed (2). Authors believed that this was due to higher viscosity of the ointment. Nevertheless, an additional explanation might exist that the volunteers could have much better control (tactile and visual) over the ointment distribution, compared with creams and lotions. The application of topical preparations, which either evaporate or are absorbed by the skin (lotions, creams), could not be as well controlled as an ointment, which usually remains at the site of application for at least several minutes.

Probably, if creams or lotions were more visible on the skin (e.g., due to added pigment, which could fade in several minutes postapplication), creams and lotions might be applied more evenly, as better control over their distribution could be achieved.

Nevertheless, there also exists a belief that the opacity of inorganic agents and the greasiness of organic agents may contribute to inadequate application of the sunscreens and subsequent reduction of their protective effect (3).

The Physical Characteristics of Preparation

If the amount of the applied topical agents was not fixed, the applied amounts strongly depended on the preparation's viscosity. For instance, a "chemical" type of sunscreen, which was easier to spread on the skin, was applied in amounts up to 50% higher than the "physical" sunscreens of higher viscosity (1.48 mg/cm^2 *vs.* 0.94 mg/cm^2, respectively (3)). Meanwhile, the same study did not reveal statistically significant differences between the amounts of the applied chemical sunscreens with various sun-protective factors (SPF): 8, 15, and 25.

Note that an earlier study (4) did not demonstrate a statistically significant difference between the amounts of applied cream, ointment, and lotion, in spite of their evidently different viscosity. Probably, this might be explained by more sensitive methods of investigations used in the later study.

Type of Container

A study demonstrated that the dispenser type might influence the amount of medication applied by the patients: the cream contained in an open jar was applied by volunteers much more readily than the same cream from a tube (1.7 mg/cm^2 *vs.* 0.71 mg/cm^2, respectively (4)).

PATIENT-DEPENDENT FACTORS

Patient-dependent, or behavioral, factors influencing dosing might be divided into three groups: (*i*) socially mediated factors; (*ii*) factors mediated by medical personnel; and (*iii*) factors mediated by patient's condition.

Socially Mediated Factors

The socially mediated factors are widely discussed regarding the use of sunscreens (although we might assume that in case of visible skin lesions, e.g., facial eczema, such factors may also play a role).

It is the social factors that may explain the fact that women used sunscreens more often than men and applied sunscreens more frequently on sun-exposed parts of the body (5,6). It should be mentioned that Centers for Disease Control and Prevention data on sun-safe behavior seemed to demonstrate that in 2005 and 2010 men reported higher levels of sun-screen behavior than women (7) (compared with that in year 2000, when sun-safe behavior was found to be more frequent in women in the U.S.A.). Nevertheless, Katz et al. (8) suggested that such a change of gender pattern might be only due to methodologic issues (the questionnaires changed significantly in 2005 and 2010). There is also an evidence that barrier creams were more often used by female workers rather than males (9).

Some associations were found between use of sunscreens and also self-tanning products by undergraduate students and their close relatives (for facial sunscreens) and romantic partners (for self-tanning preparations) (10). Household members' encouragement increased the frequency of sunscreen use by postal workers of Southern California (6). Additionally, it was demonstrated, that people whose relatives had a skin cancer history applied the sunscreens more readily than people without a family history of skin cancer (6). College education and older age were also associated with higher use of sun-protective creams (8).

Nevertheless, another study (11) demonstrated no significant gender difference in the amount of sunscreen cream applied by students from several European countries – these amounts were uniformly low, the median quantity was 0.39 mg/cm^2, while the amount needed to obtain the nominal SPF must be 1.5 mg/cm^2 [according to the requirements of the German standardization authority – Deutsches Institut für Normung, (DIN)] or 2 mg/cm^2 [according to the requirements of American Standard Association (ASA)].

No gender differences were found in the study of application of pharmaceutic topical agents (4), probably because the use of medications was perceived as necessary to the patient on the quantity of the agent to be applied per unit of time (12). Other investigators demonstrated that the assistance of medical personnel may change the amount of preparations applied by the patients.

The amount of topical medication (cream) applied by nurses was significantly less (0.91 mg/cm^2) than the quantity of cream applied by patients themselves (1.71 mg/cm^2) (4). The same authors demonstrated no significant differences between the amounts of topical preparations applied by uninstructed and instructed patients.

Operator-assisted (e.g., nurse) total-body application of cream/ointment resulted in less cream applied, but the cream was spread more equally than in the case of self-application (13). It was interesting to compare the distribution of the cream/ointment applied by patients themselves at different anatomic sites and the surface areas of the sites (Table 23.1). Significant differences existed between cream distribution over the region and the real surface of the region, for example, genitoanal area received more than sevenfold the amount of cream in comparison with its surface share.

The application of sunscreens and barrier creams, on the contrary, did reveal the influence of instructions provided by medical personnel.

Although sunscreens are supposed to be used with no instructions or assistance, Azurdia et al. (14) showed that self-applied sunscreens are spread in low mean amounts with great variability: from 0 to 1.2 mg/cm^2 at different anatomic sites (mean amount 0.5 mg/cm^2). Maximum thickness was found on the forehead, cheeks, nose, and chin (1.0 mg/cm^2 or greater), whereas the mean cream thickness on the temple, ears, lateral, and posterior neck approached zero.

TABLE 23.1
Distribution of Cream/Ointment by Body Areas. Surface of Body Area of Adults

Part of the Body	Cream Amount, %	% of Total Body Surface
Head	7.6 ± 2.3	9
Arms	22.3 ± 1.6	19
Legs	43.6 ± 23	40
Trunk, anterior	14.1 + 2.0	13
Trunk, posterior	12.4 ± 1.8	18
Genitoanal area	7.4 ± 1.0	1

Source: From Ref. 13.

Loesch and Kaplan (15) also showed that periorbital areas, perioral regions, and the ears are rarely covered by sunscreen properly; they suggested some rules for patients to enhance complete sun protection. Subsequently, Azurdia et al. (16) demonstrated that after special instructions the same patients applied 5- to 10-fold higher amounts of sunscreen than prior to instructions.

There was a thorough investigation of distribution of self-applied industrial protective cream in three groups of workers — metal workers, hospital cleaners, and construction workers (17). Many areas of the hands and forearms were not covered properly by protective cream, which might result in a higher incidence of irritant dermatitis. The authors suggested a special educational program for workers to ensure the proper use of protective cream at the workplace. Other study revealed that 28% of metal workers denied any use of skin protective measures at work, although some of them did suffer from skin problems (9).

Subsequent investigations showed that the uptake of barrier creams was significantly higher among German bakers, who received special training on skin protection, compared with those who did not receive it (18).

FACTORS DEPENDENT ON PATIENT'S CONDITION

Severe cases of skin diseases may require larger amounts of topical medications, and this is well understood by the patients, so that the patients with more severe cases are ready to apply much larger amounts of topical agents.

It was also demonstrated that, for example, the postal workers with higher skin sensitivity to sun used sunscreens significantly more frequently than their colleagues with lower skin sensitivity (6). German researchers found that metal workers with past or present history of hand eczema used both barrier creams and moisturizers significantly more often than the workers without skin problems (9).

CONCLUSION

Numerous factors may influence the applied amounts of topical preparations. It is important that the mentioned factors be considered if any conclusion is to be made on effectiveness of a topical preparation – either for a specific patient, or for a group of patients.

The investigations failed to demonstrate the influence of medical instructions on self-application of topical medications

(4); but in case of preventive agents (barrier creams and sunscreens) such instructions were beneficial (15). Further studies may provide additional information on the reasons for such a difference.

When clinical trials of topical agents are conducted, and no direct measurement of the applied amounts of the substances is provided, there should always be a proper control over possible factors influencing the application of the substances on the skin. Otherwise, the obtained data may not be comparable.

Generally, we believe that there remains much room for innovations, which would be able to provide more precise dosing of topical preparations.

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24 Immune reactions to copper

Jurij J. Hostynek

INTRODUCTION

The last decade has seen a marked expansion in interest in metal allergic contact dermatitis (ACD)—from a focus mainly on nickel and chromate to currently gold, cobalt, palladium, and others. Orthopedic implants provide the impetus for much of this interest. Case report methodology now is much of the literature citations in this area. Here we critically review the citations and suggest diagnostic criteria that might clarify how hypersensitivity to copper often occurs in man.

The skin is a target organ and indicator for allergy. Although the stratum corneum (SC) is a partial barrier to the passive penetration of allergens, to electrophilic, protein-reactive metals in particular, live tissue of the epidermis and dermis actively process penetrants or systemically absorbed allergens, which reach it. Such immune reactions to chemicals in the skin are broadly categorized into two distinct classes:

a. ACD or delayed-type reactions mediated by allergen-specific T lymphocytes. It expresses as a wide range of cutaneous eruptions upon (a second) dermal contact or systemic exposure to haptens in individuals with preformed cellular immunity (type IV allergic reactions).
b. Immunologic contact urticaria (ICU) or immediate-type hypersensitivity, which involves IgE antibody. The latter most notably results in respiratory allergy, but can also manifest in separate stages collectively described as "contact urticaria syndrome" (1); local or generalized urticaria, urticaria with extracutaneous reactions, such as asthma, rhinoconjunctivitis, and gastrointestinal involvement, and ultimately anaphylaxis (type I reactions).

Copper has been reported to sensitize de novo on systemic exposure following inhalation or implantation. The resulting dermatosis thus induced is described as systemic contact dermatitis or urticaria (2). Copper complexes are also known to elicit skin reactions upon systemic challenge in the previously sensitized organism (3).

Metallurgy of Copper and its Alloys, and its Role as a Sensitizer

Dissimilar metals, combined in alloys for the fabrication of medical devices, such as dental materials, evoke currents in electrolytic media, such as saliva and degrade, resulting in a steady release of metal ions. In immediate proximity of dental restorations or copper intrauterine devices (IUDs) this can lead to adverse (intraoral or intrauterine) reactions, such as lichenoid lesions of the oral or genital mucosa. Beyond local effects at the implant site, ions can be transported into distal tissues, such as the skin, giving rise to pathologic processes, such as manifest allergic reactions. Among the metals which commonly form allergenic ions are nickel, cobalt chromium, and mercury. Exposure type, duration, and environmental conditions (sweat, oxygen supply) in proximity of the metal are critical for mobilization of ions leading to induction or elicitation of immune reactions. As most articles of common human contact are alloys and not made of the pure metal itself, electrochemical interaction between components are significant for the release of allergenic ions potentially leading to immune reactions (4).

Reports of copper as immunogen are few, and rarely could the clinical relevance of copper sensitivity be demonstrated with certainty. Consequently, the question as to incidence or prevalence of copper sensitivity among the general population is moot, the number of cases too low to express as percentages. Nevertheless, two characteristics of copper in contact with tissues put the metal into a category that renders appropriate a discussion of its role in inducing reactions in the immune system.

- Copper belongs to the family of electrophilic transition metals, which makes the copper ion highly protein reactive, that is, likely to be haptenized, thus recognizable by the immune system as nonself or foreign.
- Although belonging to the nobler metals highly resistant to corrosion (oxidation, dissolution), in the physiologic environment (as IUD, dental materials, implants) or in contact with skin exudates, elemental copper is converted to diffusible forms which, albeit slowly, can penetrate biological membranes.

This latter factor merits detailed discussion, also to lay the groundwork for demonstrating how copper and other metals eventually become biologically available from contact with endothelial and epithelial barriers.

The oxidation of copper in body fluids has been investigated as a factor, which may determine induction or elicitation of immune reactions. Release of metal ions experimentally determined in synthetic body fluids may not adequately mimic the degree of corrosion (oxidation, release) as it occurs in contact with live skin or in the physiologic environment, however. This is because the composition of such media used for routine experimentation lacks important components which, in contact of foreign materials with a living organism determine the nature of reaction product, the rate of reaction, and thus the path of diffusion of the end products through biological barriers.

These formulas appear to omit important factors, for instance those present in skin exudates, which can play a determining role in metal oxidation: proteins (5), and, most importantly, free fatty acids in the sebum (6). Together with metal ions the latter are likely to form lipophilic soaps, presumably diffusible via the intercellular lipid matrix of the SC. Evidence for skin diffusivity in a model experiment was obtained by in vivo application of copper oleate over 24 hours on human back skin. Urinary copper levels were subsequently seen to increase significantly over several days (7). While there is a good indication of facilitated permeation, that result in itself does not indicate the actual path followed by the permeant, however. Evidence for an actual path of diffusion was obtained in a different experiment; localization of copper in the intercellular spaces was made visible through electron microscopy after the application of copper acetate on human skin (8).

Human plasma or serum are the most corrosive physiologic media and can play a decisive role on the path toward systemic immunization. Comparative tests simulating corrosion of implant metals in vitro demonstrated that the electrochemical process of oxidation in the presence of enzymes, proteins, and other components of actual serum is accelerated in comparison with standard simulating media (9). Corrosion testing of implants thus becomes more relevant for in vivo conditions when it is conducted in a proteinaceous medium (whole blood, serum, saliva).

The present synopsis of hypersensitivity cases arising from contact with copper amply, albeit indirectly, confirms the diffusivity of copper derivatives through biological barriers. In addition, that copper derivatives (abietate, naphthenate, oleate, sulfate, 8-quinolinolate) used as pesticides are reported to act as irritants when coming in contact with the skin, evidence for their diffusion beyond the SC, reaching the live strata of the skin (10).

The practice of using copper compounds, including metallic copper, as patch test materials for diagnostic purposes in dermatology also is based on empirical evidence gathered for their diffusion to reach the live strata of the epidermis when applied under occlusion.

Finally, conversion of copper metal to diffusible compounds has been demonstrated in our laboratory in a semi-quantitative manner (unpublished data). The SC of human volunteers was analyzed in depth for copper content following the application of finely distributed metal on the skin under semi-occlusive conditions. After application of the metal as micronized powder on the volar forearm for periods up to 72 hours, inductively coupled plasma mass spectroscopy analysis of sequential tape strips showed that the gradients of copper distribution profiles increased proportionally with occlusion time, from 24 to 72 hours, rising to 10 ppm after the longest period, significantly above the initial background level of 2 ppm.

Predictive Immunology Test Results for Copper

Thus far, copper has been tested for sensitization potential in two predictive tests: the Guinea Pig Maximisation Test (GPMT), a standard method used as predictor of skin sensitization potential (11), and in the Local Lymph Node Assay (LLNA) (12).

In the GPMT on 20 guinea pigs Boman et al. noted two positive reactions at 24 hours and seven at 48 hours after using 1% copper sulfate ($CuSO_4$) pentahydrate in Petrolatum (pet) (13). Karlberg et al. later found no difference between copper-exposed and control animals at 1 – 0.1% $CuSO_4$ in pet. (14). Basketter et al. obtained a 0% response in the same test (15), but later in the LLNA the result was positive (16).

In the LLNA adapted to test for allergenicity of metal salts also, under modified conditions cupric ion significantly increased lymph node cell proliferation. Testing of cupric ion as the chloride in dimethyl sulfoxide at 1, 2.5, and 5% concentrations showed significant increases in Lymph Node Cell (LNC) proliferation, with ratios of test to control lymphocyte proliferation of 8.1, 13.8, and 13.6, respectively. Also, mice could be sensitized in the LLNA by application of copper(II) sulfate (17). When the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods tested cuprous chloride in the LLNA, that copper salt was also found to increase lymph node cell proliferation, resulting in a positive test reading (18).

Diagnostic Tests for Hypersensitivity

A differential diagnosis of chemically induced urticaria (ICU), immediate-type irritant [non-ICU (NICU)], and ACD is sometimes difficult, particularly when dealing with strong irritants. In simplest terms, it is mainly based on concentration of the xenobiotic (agent) necessary to induce a skin reaction, and on the time course of reaction.

The Open Test

The material is applied to intact skin or slightly dermatotic skin, with wheal and flare developing in minutes, a positive indication of NICU or ICU (see above).

The Skin Prick Test for Immediate-type Allergy (Contact Urticaria)

Sensitization is defined as a positive skin prick test (SPT) response with or without clinical symptomatology. One drop (20 µL) of putative allergen in an appropriate solvent (e.g., propylene glycol), vehicle (negative control), and histamine in physiologic saline (positive control) is placed on three separate sites on the volar aspect of the forearm. Using a sterile prick device inserted through the drop, the underlying superficial epidermis is gently pricked. One needle is used per skin site and discarded. Immediately after pricking, each skin site is blotted dry. After 15–30 minutes the skin sites are evaluated for wheal and flare response. An edematous reaction (wheal) of at least 3 mm in diameter, surrounded by a flare, and at least half the size of the histamine control is considered positive in the absence of such a reaction in the vehicle control. SPT positives are re-tested to confirm the response. Ultimately, diagnosis should be based on clinical history and negative controls. Unlike with the open test, controls are mandatory.

Radioallergosorbent Test

Immediate allergic hypersensitivity can be diagnosed by radioallergosorbent test (RAST), an in vitro immunologic procedure designed to detect specific IgE antibodies in serum (19). Initially, a hapten–protein conjugate between a reactive compound and human serum albumin (HAS) has to be synthesized for the radioimmunoassay. The allergen (hapten–protein conjugate) is coupled to a paper disk. IgE antibodies in a serum sample, which are specific for the conjugate bind to the conjugate epitopes on the disk, and the portion of bound IgE is detected by I^{125}-labeled

antihuman IgE. Usually the results are expressed as a percentage of the total activity, the ratio between the binding to the hapten–HAS disk and a disk onto which HAS had been coupled and run in the same experiment.

The RAST Inhibition Test

Also cross-reactivity of various haptens can be determined by the RAST method. Serial dilutions of conjugate are allowed to react with an individual's serum. The mixture is then used for RAST determination. The degree of reduction of the serum RAST values after absorption is expressed as percent inhibition.

The Patch Test for Delayed-type Allergy (Allergic Contact Dermatitis)

The key diagnostic tool for ACD is patch testing. The objective is to reproduce the skin reaction to a suspected allergen under controlled conditions, by dosing the substance (or a standard series of allergens) in a suitable vehicle at a nonirritant concentration on adhesive tape and placing it on the skin. Penetration through the SC is promoted by airtight occlusion. The test is left on for at least 48 hours. Some agents elicit reactions only after a substantial delay (late phase reactions), possibly due to their anti-inflammatory activity as is the case for copper (20), or poor skin diffusivity.

It is suggested that to resolve doubtful cases a more differentiated approach using dilution series is advisable for diagnostic purposes. This would include using a dilution series rather than the standard practice of applying the single 1 or 2% $CuSO_4$ solution for patch-test assessment of allergic response to the metal salt.

The detection of allergens by patch testing with salts dissolved in water, dispersed in petrolatum, or in their elemental form, and subsequent removal of the allergen resulting in clinical improvement is the simplest and most direct connection between cause and effect. As described in greater detail below, a confounding factor in etiology and diagnosis for a number of transition elements, and particularly in the case of copper, is the well-documented cross-reactivity with other metal ions, primarily nickel, but also palladium. These are reactions occurring when haptens of similar size and electron shell configuration are transferred to the same carrier protein. In fact, in the majority of copper sensitivity cases reported the patients, when tested for multiple metal allergies, were positive to two or more metals, nickel being the most frequent one (21). That is an allergen which can induce clinical manifestations in even minute amounts, and is ubiquitous in the normal environment. Thus false-positive reactions to copper may be due to the presence of trace contaminants in the putative cause for allergy, for example, the IUD, or even in the diagnostic test material, for example, the metal disk (also see below).

Because of the inherent irritancy of $CuSO_4$ under patch-test occlusion, and the relatively small number of normal volunteer controls used, we estimate that the nonirritating dose for diagnostic testing approximates 1–2% in petrolatum. For verification of copper-allergic hypersensitivity, application of 1% $CuSO_4$ in water or petrolatum is recommended by the International Contact Dermatitis Research Group (ICDRG), or of an occluded copper (metal) disk over 2–4 days. Petrolatum is the recommended vehicle for $CuSO_4$, although uniform distribution of the crystalline salt is problematic, and poor penetration from petrolatum makes that choice less than ideal. Any positive reactions warrant further evaluation to ascertain clinical relevance (22).

One alternative method advocates the use of metals in the elemental state for diagnostic skin tests, and several authors have used copper disks or currency for patch test. The diagnostic value of this approach is put in question by one investigation, however, where metallic copper was immersed in synthetic sweat to analyze for metal release. Over 24 hours the final copper concentration was 0.01%, considered by the authors to be too low to elicit a reaction except in highly sensitized individuals (23).

Role of Vehicle in Patch Testing

In choosing a vehicle for percutaneous penetration, a factor for consideration is the effect it will have on the skin membrane and thus its barrier properties, since the solvent of a xenobiotic (metal) can significantly influence its diffusivity and thus bioavailability. Petrolatum for instance is a poor solvent for metal salts, where the permeant remains suspended as fine particles affording less-than ideal uniformity in skin contact, but on the other hand has an occlusive effect which would increase skin hydration and thus promote diffusion of a hydrophilic compound. Another solvent which enhances penetration is dimethylsulfoxide (DMSO). As an instance, Sharata and Burnette point to dimethyl formamide and dimethyl acetamide associated with DMSO, which cause swelling of basal SC cells and disrupt the normal keratin pattern. They located the electron-dense metal ions mercury and nickel in the intercellular spaces and corneocytes, whereas in control membranes those metals were seen almost exclusively in the intercellular space. Thus certain solvents may modify intercellular solute diffusion to include the transcellular path (24). How the nature of the vehicle can either influence the rate of release of a compound or modify the barrier properties, thus determining the level of percutaneous absorption of xenobiotics is illustrated by further examples from the literature. An instance of practical importance is the choice of vehicle in standard diagnostic skin patch testing for sensitization, with the aim of optimum release of allergen into the viable epidermis while avoiding allergic or irritant contact dermatitis leading to false-positive reactions caused by the vehicle itself.

Poorer penetration of salts formulated in petrolatum was demonstrated repeatedly. Fullerton et al. explored the effect of water as vehicle for $NiC1_2$, and of petrolatum for both $NiC1_2$ and $NiSO_4$ at l.32 mg Ni/mL through in vitro experiments with full-thickness human skin (25).

From experience in dermatologic practice, particularly in consideration of the clinical picture emerging from the few cases that document copper allergy, and because of the complexity of the irritant dermatitis syndrome, adhering to the criteria set out in the Operational Definition of Allergic Contact Dermatitis (22) is recommended when a definition of clinical relevance is sought.

Test Concentrations for Copper ACD

Since relatively few dermatotoxicologic investigations have researched copper characteristics as allergen, no definite value has been assigned as to $CuSO_4$'s threshold-inducing sensitization, nor is an optimal concentration defined, which will reliably elicit reactions in the sensitized organism. Thus the patch test doses vary: 1–2% pet.; in a dental screening tray concentrations include 1 and 2% aq. (26).

Immunogenic Potential of Copper

Systemic Allergic Contact Dermatitis

Systemically induced allergic disease, which can be caused by T-cell-mediated reactions to metals, such as copper (27), potentially occur when copper or copper-containing alloy materials used in IUDs, implants in replacement surgery or orthodontic appliances, are oxidized with release of free copper ions. These are absorbed through the epithelia and carried to the skin and the mucosa via blood and the lymphatic circulation. There the allergen is intercepted by antigen-presenting cells and recognized by T cells, which migrate to the lymph nodes with blastic transformation, proliferation of cytotoxic lymphocytes, and production of cytokines. These in turn recall neutrophils and eosinophils to the reaction site, cause capillary dilation and increased permeability, resulting in cutaneous inflammation appearing as wheal and flare. Lichen planus and asymptomatic contact hypersensitivity (dental alloy contact dermatitis) are increasingly being linked with oral exposure to materials used in dental fillings, orthodontic prostheses, cements, and components of dentures, bridges, bands, and wires. Such reactions can be either immunologic contact stomatitis, or systemic anaphylactic stomatitis (type I reactions), or delayed contact stomatitis (type II). In a few instances copper was implicated as the possible cause for the latter (28), as copper is commonly part of alloys used in dental materials. In a study investigating the release of copper from a selection of orthodontic appliances in organic and inorganic solutions made up to different pH values to imitate the oral environment, Stoffolani et al. found that the levels of metal mobilized were well below those ingested with a normal daily diet. From that result they concluded that the quantities released should be of no concern. The relevance of that conclusion, particularly for purposes of immunology, invites further discussion, however, to be pursued elsewhere (29).

A study of professionals involved in making and handling such materials: dental technicians, orthodontists, and their assistants, reveals that in handling dental devices they also run the risk of developing hypersensitivity to allergenic materials, metals among them, as in one study 40% of orthodontists and 43% of dental assistants reported work-related skin problems (30).

Copper Intrauterine Devices

Copper metal in contact with biological substrates (as in IUDs) is highly reactive and releases free copper ions. Release in vivo was determined at 0.71 I-1 mol/day (45 I-1 g) from a surface area of 200 mm sq. and 1.29 I-1 mol/day (82 µg) in a culture medium in vitro (31).

After it was discovered that copper metal placed in the uterus of animals has a contraceptive effect (32), the principle was applied to humans: a plastic T-shaped device with copper wire or a copper sleeve was introduced as a pharmacologic agent and became widely used as an intrauterine device to regulate fertility. Research suggests that copper prevents fertilization rather than implantation (33).

Dual Immune Response to Copper

Although organic compounds infrequently cause both types of reactions, dual immune response appears more common for metals and metallic compounds. Their reactivity toward protein results in a complete antigen, which triggers both IgE antibody production (type I) and cellular (T cell, type IV) immune reactions. Immunogenic effects that result from exposure to metals can be attributed to the same factors that determine their toxicologic and biological effects. Metal ions in general, and certainly those belonging to the transition group of elements, such as copper, have an ionic radius too small to be antigenic. Containing a partially filled d-shell, however, these metals oxidize to highly electropositive cations, which can act as haptens interacting with tissue protein. They form bonds which range from fully ionized to fully chelated complexes, and have the ability to modify the native protein configuration. These are recognized as nonself by hapten-specific T cells in the host immune system, leading to allergic reactions of the two different types (1–3).

Copper is one of the several metals causing more than one type of hypersensitivity presenting with multiple symptoms in allergic responses, in part depending on type of exposure: immediate type, ICU sometimes associated with respiratory hypersensitivity, delayed-type cutaneous hypersensitivity, systemic allergic reactions, as well as contact stomatitis. Such concurrent occurrence of immediate and delayed-type sensitivity has also been observed in the same individual (38).

Recommended Patch-Test Procedure in Suspected Copper Allergy of the Delayed Type

- Establish clinical history (anamnesis) determining the nature of contact and physical form of putative allergen
- Physical examination of the patient
- Patch testing with 2% $CuSO_4$ in petrolatum. In case of positive outcome followup with serial dilution patch testing (1, 0.5, and 0.1%).
- Repeat open application test (ROAT) or provocative use test (PUT) with a dilution series of $CuSO_4$: 2%, with at least 10 naïve control subjects to demonstrate that positive reaction was not irritant in nature, then further at 1 and 0.5%. The substance is applied once or twice daily for 14–28 days (34). A positive reaction usually appears within four days, less frequently between five and seven days. Delayed reactions have been noted in patch testing with copper.
- To confirm positive patch-test reactions and identify false-negative reactions on patch testing, intradermal tests may be considered. Herbst et al. provide the scientific background of intradermal testing for ACD (35).
- As an "alternative" predictive test for ACD, the local lymph node assay was developed on mice for the detection of contact allergens (36). It has been adapted to test for allergenicity of metal salts also. Under modified conditions, cupric ion was seen to significantly increase lymph node cell proliferation, as mice could be sensitized by application of copper(II) sulfate (37).

Recommended Screening Procedure in Suspected Copper Urticaria

- Open test: Application on healthy skin first and observation of the test area for 60 minutes. If reaction is negative, on previously affected skin (as suggested by patient's

anamnesis) spreading of 2% aq. $CuSO_4$ on a 3 × 3 cm area. Immunologically mediated reactions usually appear within 15–20 minutes, nonimmunologic ones within 45–60 minutes after application (1). This difference in delay is a major distinction between specific and nonspecific contact urticaria. A positive reaction is seen as edema or erythema (wheal and flare). A minimum of 10 naïve background controls with the test solution is suggested. A nonimmunologic reaction will appear in the controls due to release of inflammatory mediators from the cells without participation of specific IgE antibody (10).

- A use test is suggested, handling the suspected agent and re-creating the original scenario inducing the reaction (34).
- When open application is negative, a prick test with 2% aq. $CuSO_4$ is suggested. A group of more than 10 background controls is required in prick testing using physiologic saline solution to ascertain that copper does not produce such lesions in normal controls.
- The occluded application of a copper disk over 48 h can also confirm suspected sensitization.
- In case of a positive test the open application may be repeated for verification.

Confounding Factors in Copper Allergy Test Results: Cross-reactivity, Contaminants, Irritation, and Angry Back Syndrome

In many cases where copper allergy is suspected, positive patch tests to copper (as metal or the sulfate) are equivocal, and assignment of clinical relevance can be difficult or impossible because case reports in the literature most often lack relevant details. One element of uncertainty in the diagnosis of copper allergy is its cross-reactivity with other (adjacent) transition metals in the periodic system of elements. Observations of multiple sensitivity to metals have been made frequently, attributed to cutaneous or systemic contact with alloys, and it is challenging for the investigator to ascribe the clinical observation either to concomitant sensitization or to cross-reactivity. Often patients react to compounds that are not the primary sensitizers. Originally, Epstein had raised the question of nickel and copper cross-sensitization in 1955 (39), and since then many cases of simultaneous sensitivity to nickel and copper in the same organism have been reported. The immunologic mechanism involved in hypersensitivity to multiple metals and cross-reactivity between copper and other transition elements has been investigated in two independent in vitro studies and the event is well characterized now, making it possible to put the numerous case reports on copper-induced allergy in better perspective. Specifically, nickel ion-specific T-cell clones appear to be recognized both by copper and palladium ions, but not by others, such as cobalt. This reactivity is likely to be favored by their bivalency and proximity to nickel in the periodic table of elements. Investigations showed that among a large panel of nickel-specific T-cell clones four different types of reactivity can occur: reactivity to nickel only, cross-reactivity between nickel and palladium, cross-reactivity of nickel to copper, or to both palladium and copper ion, which both neighbor nickel in the periodic table of elements (40). In light of these results, copper-positive patients are now more often screened for allergy to other metals also, but only few among them are found to be truly copper-sensitive.

Purity of test materials can be a source of diagnostic equivocation with the potential for false-positive results. Copper patch-test material may contain nickel as an impurity, as analytical grade $CuSO_4$ was shown to contain up to 0.002% nickel; high-purity copper wire in IUDs, which is also used for skin testing contain 0.0003% (3 ppm) nickel. Note that with metal ACD in humans, highly sensitized subjects can react down to a few parts per million of the hapten (41).

A potential cause of false positive, clinically nonrelevant reactions, which can result in patch testing is hyper-reactive skin, also known as the Excited Skin Syndrome or "Angry Back" (42). This condition can result from multiple inflammatory skin conditions or from strong positive patch-test reactions, magnifying adjacent patch-test responses or inducing nonspecific reactions. This is a potential occurrence in testing for copper when several different metal patches are simultaneously applied on the patient. Multiple positive reactions may require separate, sequential tests with the involved substances.

Finally, several studies, especially those involving retrospective reviews or large population groups routinely examine skin reactions at 48 hours, missing potential late-phase (72 hours) reactions after patch application. They may result in false-negative diagnoses and under-reporting of hypersensitivity to copper.

Determining Clinical Relevance

The open literature has been critically reviewed for clinical relevance of the cases reported. A problem encountered often in the evaluation of diagnostic tests from patients reacting to chemical substances is understanding the clinical relevance of test results, because little or no data are reported to qualify positive results. This becomes particularly difficult in the interpretation of tests that appear to indicate a compound as primary sensitizer, which is known to have no or little sensitization potential, such as copper. Benezra et al. have addressed the problem of classification by suggesting a systematic analysis of available data to arrive at an expression of degree of confidence in the results reported by investigators, thus to better define morbidity of a putative allergen. A degree of confidence was assigned to all cases listed with the literature reports. Although Benezra et al. designed the system with skin contact sensitizers in mind, which lead to delayed-type reactions, the approach appears more generally valid and is applied to all cases reviewed here (43).

Criteria for Assignment of Degree of Confidence

- Presence of vehicle-treated or -untreated controls
- Concentration of test substance judged sufficient to elicit response
- Use of an appropriate vehicle
- Purity of test reagent to exclude possible reaction to contaminants
- Sufficient number of cases for meaningful response

The evidence provided in the reports is evaluated toward classification of the agent (copper) as allergen and a degree of confidence

on a scale from 0 to 5 is assigned to indicate how well the test results demonstrate that the chemical does or does not induce the immune reaction:

5 = Results meet all of the criteria
4 = All criteria met, but number of cases is marginal
3 = Parameters such as controls are missing but reports point to substance as sensitizer
2 = Controls are absent and there are no other details indicating substance as sensitizer
1 = Results not considered to be reliable
0 = Test fails all of the criteria

Since evaluation of criteria is subjective, degree of confidence should be viewed within a range of ±1 of the number assigned. Listed in the following are reports relative to copper hypersensitivity: population-based studies, selected from published reports of immune reactions to copper, which surveyed larger samples: random cross-sections of the population, cohorts of specific occupational exposure, wearers of IUDs, dermatologic clinical databases, or groups exposed to copper in dental materials.

Summaries of Studies, Including Population-Based Studies

Barranco, 1972

Upon review of the literature the author noted six cases of ACD to copper: cases attributed to contact with brass, and one each to exposure to $CuSO_4$, copper metal, and jewelry. The author also reports on a case of dermatitis attributed to the use of a copper IUD. Although of questionable clinical relevance due to patch testing with 5% $CuSO_4$, it holds a somewhat historical interest as it is the first report of eczematous dermatitis to copper due to systemic exposure. Tested for the other frequent metal allergens: Ni, Cr, Co, and Hg besides Cu, all patch tests were negative except for a strong reaction to 5% $CuSO_4$. Remission was noted after removal of the IUD (44).

Dhir, 1977

A cohort of 10 furniture polishers who had developed skin reactions on handling ethyl alcohol tinted with 5% $CuSO_4$ were tested with that solution and aq. 5% $CuSO_4$. All 10 patients reacted to both materials; the test with the same materials were negative on 15 control subjects (45).

Jouppila, 1979

Assessed were 37 patients wearing copper IUD and presenting with skin rashes. Epicutaneous tests for copper, nickel, and cobalt allergy showed reactions to nickel (4) and cobalt (1), but none to copper. The authors concluded that allergy to copper was not likely to be the cause of the side effects (46).

Karlberg, 1983

Of 1190 eczema patients tested with serial dilutions (2–0.125%) $CuSO_4$ in pet. over a three-year period, none had a reaction to copper only, 13 reacted to copper and other metals. Thus no sensitization to copper specifically became evident, leading to the assumption that the (multiple) reactions noted were due to metals contaminating the test allergen. According to Karlberg, highest-grade copper metal contains 0.0003% nickel, analytic grade $CuSO_4$ up to 0.002%. In the GPMT using dilution series of 0.1–0.01% $CuSO_4$ for induction and 1–0.05% in pet. for elicitation, Karlberg determined that $CuSO_4$ was a grade 1 allergen. In her review of the literature prior to 1982, Karlberg noted four relevant and 20 probably relevant cases of copper hypersensitivity. Over 90 cases were classified as uncertain or not relevant (14).

Lisi, 1987

The authors studied the prevalence of irritant or ACD from pesticides by patch tests on 652 outpatients with skin disorders. Of 564 subjects tested with 1% $CuSO_4$, four cases showed positive reactions, none of which were irritant morphology. The presumed allergic reactions cannot be considered of definite relevance due to scarcity of clinical details. In particular, data are missing on confirmatory re-testing of positive tests conducted two to three months later (10).

Zabel, 1990

Records on 10,936 patch-test reactions collected in a dermatology clinic over the period 1975–1985 were reviewed, in addition to patch tests conducted on 118 patients wearing IUDs. Besides the record of patients with positive reactions to multiple metals (mostly nickel), one eczematous IUD-wearing patient reacted to $CuSO_4$ at 5% in pet. only. After removal of the IUD the eczema resolved. The causative role of copper is uncertain due to lack in supporting evidence in that case (47).

Motolese, 1993

The authors report on skin sensitization to metals encountered in a cohort of enamellers and decorators. Relevance of the only positive reaction to copper is uncertain due to the high concentration of 5% $CuSO_4$ used in the test. Also, too few clinical details were given to establish a firm cause–effect relationship in that case (48).

Kawahara, 1993

The cause of occupational allergies was investigated in a dental technology school by testing a cohort of 12 students with 40 potential contact allergens occurring in the manufacture of prostheses as dust, mist, and fumes in their environment. Two reacted to 1% aq. $CuSO_4$; the reactions could not be assessed as to their clinical relevance due to lack of any further details (49).

Tschernitschek, 1998

Over the period 1982–1997 in a dental clinic, of 311 patients who were patch tested for dental materials-induced hypersensitivity, 13% showed positive reactions. Most frequent among the sensitizing materials were metals (77 of 107). Three among those reacted to copper and cadmium, two to copper only. Significance cannot be assigned due to total lack of experimental details (50).

Candura, 1999

Of 233 ACD outpatients patch tested with the standard GIRDCA test series in a dermatologic clinic, three had positive reactions to

copper along with other metals and four to copper only. The importance of the causative role of copper cannot be assessed due to a total lack of experimental details (51).

Vilaplana, 2000

A testing program including 520 patients with dental prostheses who presented with adverse oral mucous membrane reactions was conducted using a special metal test series, which included 1% $CuSO_4$ in pet. Of 289 patients with one or more positive reactions, one patient only reacted to copper, classified as a reaction of past relevance by the authors; 2 patients had reactions to copper with unknown relevance (52).

Wöhrl, 2001

In the endeavor to assess the relevance and diagnostic value of positive reactions to copper, 2660 routine patch tests recorded in an allergy clinic over 2.5 years were screened for positive reactions to copper (2% $CuSO_4$ in pet.) and the other metals in the immediate vicinity in the periodic system of elements: nickel, palladium, cobalt, and mercury. Of 94 cases which were copper positive, 26 were enrolled in a retest program involving $CuSO_4$ at 5, 2, 1, 0.6, 0.2, and 0.05% aq. Also testing with copper foil was included. Of the original 26 inductees, 10 were positive to copper on re-testing with 5% $CuSO_4$ in pet., but eight of those also reacted to a nickel patch. Two of 10 showed unequivocally positive reactions to 2% $CuSO_4$ in pet. Two were positive to copper foil. Only one case showed an isolated sensitivity to copper and not to any of the other test allergens, presenting with chronic eczema of the fingertips. That patient's occupation as electrician would characterize the case as ACD to copper induced through cutaneous contact. One other patient with multiple metal sensitivities appeared to have clinically relevant sensitivity to copper. Presenting with eczema to a golden ring (test to gold negative), the condition resolved when the patient exchanged the gold ring with one made of silver. Although authors concluded on copper–nickel cross-reactivity on the T-cell level in 9 of the 10 cases with a high statistical association and copper sensitivity being of low clinical relevance, all reactions cleared at 96 hours, a delay which is typical for irritant reactions rather than ACD (53).

Vergara, 2004

A 56-year-old woman presented with pain in the left buccal mucosa for the past five years. She showed us the report of a biopsy, done in 1998 at another hospital, with the histologic diagnosis of lichen planus. She had been treated with triamcinolone acetonide in Orabase® for three years with no improvement. For 25 years she had had a metal dental prosthesis. The patient stated that she had had the same problem with the mucosa of the right buccal muscle, but that this had resolved when an adjacent dental prosthesis had been removed. This prosthesis was the same as the one she still had on the left and both had been inserted at the same time. On physical examination there was a whitish reticulated area on the mucous membrane of the left buccal mucosa adjacent to a metal prosthesis, and depapillated areas on the left side of the tongue. There were no lesions outside the mouth. She was patch tested with the Spanish (GEIDC) standard and dental screening series (Chemotechnique®, Malmö, Sweden). The only positive reaction was to $CuSO_4$ 2% pet. (++). Her prosthesis contained copper. It was recommended that it should be changed, after which there was almost immediate relief of her symptoms and, six months later, her lesions had much improved (54).

Forte, 2008

Metal-induced ACD is expressed in a wide range of cutaneous reactions following dermal and systemic exposure to a variety of industrial and personal products featuring copper among other metals. The authors were motivated to establish the epidemiology of metal allergens, the types of exposure, the skin penetration, the immune response, and its protein interaction. Since copper has a low sensitizing potential, it is considered to be a rare cause of ACD. For this reason, the low number of cases of copper allergy did not allow to calculate the prevalence among the general population in terms of percentage. As no regulations exist limiting this metal in products to prevent copper hypersensitivity, ACD creams containing chelating agents can be adopted by sensitized patients. This is the case of 10% DTPA, which reportedly has a significant capability to abrogate positive patch-test reactions to copper (55).

Raap, 2009

In a retrospective analysis of 206 patients exposed to dental materials with manifestations of oral allergy, only one was found to have clinically relevant allergy to copper according to symptoms (56).

COMMENTS

Many case reports of sensitization attributed to copper may be difficult to classify as such with certainty. Copper sensitivity may overlap with nickel hypersensitivity, or nickel alone may even be the only causative agent, as in dermatologic or dental practice they can only be distinguished with difficulty when assessing exposure in the individual patient. As results from several indepth investigations show, patients with a positive test to copper also appear sensitized to nickel, and vice versa. This can be attributed to cell-biological and metallurgic factors:

- Investigations at the cellular level have established cross-reactivity between the two metals, which may account for the frequency of copper hypersensitivity reported.

At the exposure level, often copper and nickel are associated, in IUDs or orthodontic materials; copper of highest purity still contains traces of nickel, thus sensitization observed may be concomitant.

- In dermatologic practice, diagnostic test materials $CuSO_4$ or copper metal disks also contain low levels of nickel sufficient to elicit a reaction in an organism highly sensitive to nickel, leading to a false-positive diagnosis.

There may be true allergic reactions to copper exposure, topical or systemic: to copper salts, to the metal or to its alloys. Judging from the cases reviewed so far, such responses are rare.

CONCLUSIONS

Systemic as well as topical exposure to copper can cause both immediate and delayed-type sensitization. Contact dermatitis and urticaria attributed to copper metal or its compounds has been suggested as effects from dental materials and IUDs as the main etiologic factors. Immune reactions occurring in industry are few considering the number of copper smelters and refinery workers in daily contact with the metal. The majority of sensitization reports may be due to copper cross-reactivity with nickel and palladium. Thus true allergic reactions to copper appear rare, particularly those induced by skin contact, which is consistent with copper's rating as a grade 1 allergen in the guinea pig maximization test. Most cases of confirmed copper allergy result from its presence in orthodontic materials, and those reactions are mostly of the delayed type.

Firmer chemical and epidemiologic judgments will be possible when:

1. Additional experimental data becomes available on the nonirritating dose(s) suitable for diagnostic patch testing (in petrolatum and water), and in water for prick testing. On the basis of Wöhrl's data, 2% in petrolatum may be appropriate.
2. Authors describe their clinical experimental data with details of the several steps as documented in the Operational Definition of Allergic Contact Dermatitis, specifically: re-patch testing upon indication, serial dilution patch testing, and use testing (PUT/ROAT). Those steps will help clarify clinical relevance.

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25 Sodium lauryl sulfatex

Cheol Heon Lee and Howard I. Maibach

INTRODUCTION

Sodium lauryl sulfate (SLS) is an anionic surface active agent used as an emulsifier in many pharmaceutic vehicles, cosmetics, foaming dentifrices, and foods, and it is the sodium salt of lauryl sulfate that conforms to the formula: $CH_3(CH_2)_{10}CH_2OSO_3Na$ (1). The action of SLS on surface tension is putatively the cause of its irritancy, and its great capacity for altering the stratum corneum (SC) makes it useful to enhance penetration of other substances in patch tests and in animal assays.

Kligman (2) found no sensitization to SLS in hundred volunteers in whom SLS was employed in provocative or prophetic patch-test procedures. There are isolated reports of contact sensitization to SLS (3–5). Some important characteristics have been proposed for irritants used experimentally: no systemic toxicity, noncarcinogenic, not a sensitizer, chemically well defined, no extreme pH value, and not a cause of cosmetic inconveniences to exposed subjects (6). SLS fulfils these criteria as a model irritant in the study of experimental irritant contact dermatitis (ICD).

APPLICATION METHODS

Many studies concerned with cutaneous irritation use a 24-h patch application. A 7-h patch (7) and 4-h patch (8) with high concentration of SLS have been developed. In real life, surfactant exposure is usually of short duration, open application, and cumulative. A single challenge of skin with an irritant insult is a momentary reflection of the skin's susceptibility, which does not consider the cumulative effect of irritation or the repair mechanisms of the skin. Repetitive challenges allow for these effects. Assay methods similar to real usage situation, such as repeated short duration chamber test (9,10), repeated open application test (11–14), plastic occlusion stress test (15,16), and soak or wash test (17,18) were developed.

A correlation coefficient of 0.63 between a single exposure and a 4-day repetitive exposure to patch testing with SLS was found (19). With repeated open application of SLS for five days as well as a single 24-h patch test with SLS using small (8 mm) patch-test chambers, only the degree of skin damage caused by the repeated open test was found associated with prior skin complaints (20). Lammintausta et al. (11) observed the decrease in patch-test reactivity secondary to cumulative open SLS application using small (8 mm) patch-test chambers and suggested that the induced hyporeactivity might be one of the false-negative diagnostic patch tests. There are two contrasting responses of cumulative SLS irritation; hyporeactivity may be noted if epidermal responses, including hyperkeratosis and dryness, were major reactions to irritant; whereas, if dermal reactions, such as erythema and edema, were major components, hyper-reactivity may develop (21). Heinemann et al. (22) observed decreased response during the third week of 0.5% SLS irritation and the increase of ceramide in the first three weeks after irritation, and they suggested that ceramide 1 seemed to play a key role as a protective mechanism against repeated irritation. In a study of repeated irritation of 0.1%, 1%, and 2% SLS for three weeks, there was higher rate of hardening phenomenon in higher concentration of SLS (30%, 40%, and 60% in each concentration) and the changes of ceramide amounts after repeated irritation coincided with the hardening only in 53.3%, suggesting that hardening phenomenon is partially related to the ceramide in SC (23).

Tupker et al. (24) divided the studies on SLS into two categories with respect to aims. The first category, provocative testing, concerns studies in which SLS is used to induce a definite skin reaction in all individuals. Aims of the first category are to elucidate the mechanism of skin irritation, to predict the irritant potency of different detergents, to study the time course after irritation, to compare the sensitivity of different noninvasive methods, to compare the efficacy of different moisturizers, barrier creams, or corticosteroids in preventing or healing skin irritation. The second category, susceptibility evaluation, concerns studies aimed to predict the irritant susceptibility of individuals, and investigate individual and environmental factors determining this susceptibility. Petersen et al. (25) suggested that SLS-induced inflammation might be a useful model for studying the mechanisms of inflammatory pain.

There are some variations in skin responses to identical patch tests, and standardization of patch-test procedure is necessary to minimize the variations in patch-test responses. Tupker et al. (24) suggested the guidelines on SLS exposure tests.

Purity and Carbon Length of SLS

There were significant differences in the irritant potential in vivo for different qualities of SLS, and there were cases in which some of the C12 chains had been substituted by longer and less-irritating carbon chains (26). The presence of C12 chains of SLS is known to elicit a maximum irritant reaction (27–30). So, only SLS qualities of high purity (>99%) should be used for irritant patch testing and the quality and the purity of SLS should be stated.

Quantity and Concentration of Test Solution

Quantity of test solution is important and larger quantities of test solution give more intense skin reactions, although the concentration of the irritant is kept constant (31,32), and Agner (33) suggested that the Duhring chamber, the 12-mm Finn chamber, or even large chambers having bigger test areas are more effective in eliciting a response. Mikulowska and Andersson (34) observed

that the effects of 8-mm chambers could result in increased, unchanged, or decreased Langerhans cells (LC) numbers, whereas 12-mm chambers always produced decrease in LC numbers. Lee et al. (35) also compared the effect of chamber size on SLS irritation on the volar forearm using three different sizes (8, 12, and 18 mm) of Finn chambers. The increase in visual score and transepidermal water loss (TEWL) at the patch-tested sites with large (12 mm) Finn chamber was greater than that with the small (8 mm) Finn chamber. However, there were no significant differences between large and extralarge (18 mm) Finn chambers.

Aramaki et al. (36) studied the interrelationship between SLS concentration and duration of exposure in irritant skin reaction. The influence of SLS concentration and duration of exposure was demonstrated with a standardized coefficient value β. For TEWL, the β value of the SLS concentration was 1.5-fold higher than that found for the exposure time. For the laser Doppler flowmetry (LD), the β value of concentration was 2.5-fold higher than that found for the exposure time and they suggested that the skin reaction to SLS could be calculated by the following formulae: $\Delta\text{TEWL} = 14.36 \times \text{concentration} + 0.82 \times \text{duration (hours)} - 5.12$, and $\text{LD} = 30.81 \times \text{concentration} + 1.09 \times \text{duration} + 2.49$. This estimation is only valid for a patch application of ≤24 hours.

Brasch et al. (37) have analyzed the synchronous reproducibility of patch tests with various concentrations of SLS aqueous solution (0.0625, 0.125, 0.25, 0.5, and 1.0%) using large Finn chamber, and they suggested that 1.0% SLS aqueous solution is appropriate for an irritant patch test as a positive control. Contamination with bacteria was found in the SLS solutions of lower concentrations resulting in decreased concentration of SLS, and the storage of SLS solutions of very low concentrations should be at low temperature and preferably in sterile vials (38).

Evaporation and Temperature of Test Solution

The penetration of SLS through the skin barrier is significantly increased by the increase in the temperature of test solution (39). Berardesca et al. (40) reported significantly different skin responses to the temperature of test solution (4, 20, and 40°C). Skin damage was higher in sites treated with warmer temperatures, and there was a highly significant correlation between irritation and temperature of test solution. Ohlenschlaeger et al. (41) also demonstrated increased irritation on the application site of warmer solution using repeated immersion in an SLS solution at 20 and 40°C. Transition from a packed gel state to a more fluid crystalline state in SC lipids occurs at temperatures between 38 and 40°C, and the fluidity of SC is important in the percutaneous penetration process as an explanation of increased irritancy at higher temperatures (40). The evaporation rate of aqueous solutions from Finn chambers was reported as 1 mg/3 min (42). Evaporation from the patch before application inhibits the inflammatory response, although the relative concentration of the irritant is increased by the evaporation process (43). This inhibition of skin irritation could be the result of decreased amount or lowered temperature owing to evaporation of test solution.

Time of Evaluation

When noninvasive measurements of the skin response are made, the interval between removal of the patch and the measurement should allow for a period of increased evaporation following occlusion. Equalization of water diffusion between the SC and the ambient air is settled after 20 minutes of patch removal (44). For measurements of TEWL, in most research articles, the interval was reported to be 30 minutes (45–47). The time course of TEWL after SLS patch testing demonstrated a significant reduction in the TEWL from 30 to 60 minutes after removal of the patch, but not from 60 to 180 minutes (48), and they suggested that evaluation of irritant patch-test reactions by the measurement of TEWL can naturally be made at any time after removal of the patches, as long as the time period is precisely accounted for. Others have argued that a minimum waiting period of 2 or 3 hours should be allowed for evaporation of excessive water due to occlusion (19,49). Aramaki et al. (36) suggested that TEWL measurement performed 30 minutes after patch removal is too early and measurement 24 hours after patch removal should be done for practical reasons.

Guidelines on SLS Exposure Methods

High-purity (>99%) SLS must be used in any study, dissolved water in occlusive and open testing, while tap water may be acceptable in immersion testing. Standard-sized occlusion chambers with filter paper disks corresponding to large (12 mm, 60 μL) and extralarge (18 mm, 200 μL) Finn chamber are recommended. The extralarge Finn chambers are recommended for repeated applications. For open exposures, 20 mm diameter plastic ring is advised. The volume of the solutions must be such that the total exposure area is covered (800 μL). Chambers should be applied to the skin immediately, that is, within 1 minute after preparation with the test solution. TEWL measurement should be performed a minimum of 1 hour after removal of test chambers. European Society of Contact Dermatitis (ESCD) proposed new guidelines in terms of purposes and methods of SLS exposure test (Table 25.1).

BIOLOGICAL ENDPOINTS

Clinical Appearance of SLS Reaction

Erythema, infiltration, and superficial erosion can be seen during acute reaction to SLS. With higher concentrations, vesicular and pustular reactions may be seen. During healing of acute reactions, scaling and fissuring will take over. The same appearance of erythema, scaling, and fissuring is seen during repeated application of SLS. The soap effect consisting of fine wrinkled surface or chapping is not commonly seen in SLS patch-test reaction (24). The modified visual scoring system of Frosch and Kligman (9) has been used to evaluate clinical skin reaction to SLS in many studies reported in the literature. Tupker et al. (24) developed the guideline concerning the visual scoring schemes for the acute and cumulative reactions to SLS (Tables 25.2 and 25.3).

Pathogenesis of SLS Reaction

The histopathologic changes induced by SLS depend on various factors, including concentration, mode of application, and time of evaluation. Acute reaction to SLS application in epidermis can include hyperkeratosis, parakeratosis, spongiosis, intracellular edema, hydropic degeneration of basal cell, and necrosis (50–53). In dermis, there were variable degrees of inflammatory cell infiltration, edema, and collagen degeneration. T lymphocytes are the predominant infiltrating cells and CD4(+) cells outnumbered the CD8(+) cells (54–58). The histologic changes to cumulative SLS

TABLE 25.1
ESCD Guidelines on SLS Exposure Tests with TEWL Measurement

	Susceptibility evaluation		Provocative testing	
	acute	**cumulative**	**acute**	**cumulative**
One-time occlusion test		Not	24 hr	Not applicable
Application time	24 hr	applicable	Chamber 12 mm	
Mode of application	Chamber 12 mm		2%	
SLS w/v%	0.5%			
Repeated occlusion test		2 hr once daily	Not applicable	2 hr once daily
Application time	Not applicable	3 wk[a]		3 wk[a]
Application period		Chamber 18 mm		Chamber 18 mm
Mode of application		0.25%		1%
SLS w/v%				
Open test		10 min once daily	Not possible[a]	10 min once daily
Application time	60 min twice daily	3 wk[a]		3 wk[a]
Application period	1 day	20 mm guard ring		20 mm guard ring
Mode of application	20 mm guard ring	1%		1%
SLS w/v%	10%			
Immersion test[b]		10 min twice daily	30 min twice daily	10 min once daily
Immersion time	30 min twice daily	3 wk[a]	1 day	3 wk[a]
Application period	1 day	Forearm immersion	Forearm immersion	Forearm immersion
Mode of application	Forearm immersion	0.5%	2%	2%
SLS w/v%	0.5%			

[a]One week is 5 application days.
[b]Water temperature 35°C.
Abbreviations: ESCD, European Society of Contact Dermatitis; SLS, sodium lauryl sulfate; TEWL, transepidermal water loss.
Source: From Ref. 24.

TABLE 25.2
ESCD Guideline on Clinical Scoring of Acute SLS Irritant Reactions

Score	Qualification	Description
0	Negative	No reaction
1/2	Doubtful	Very weak erythema or minute scaling
1	Weak	Weak erythema, slight edema, slight scaling, and/or slight roughness
2	Moderate	Moderate degree of erythema, edema, scaling, roughness, erosions, vesicles, bullae, crusting, and/or fissuring
3	Strong	Marked degree of: erythema, edema, scaling, roughness, erosions, vesicles, bullae, crusting, and/or fissuring
4	very strong/caustic	Similar to score 3, with necrotic areas

Note: Reading 25–96 hr after one-time exposure.
Abbreviations: ESCD, European Society of Contact Dermatitis; SLS, sodium lauryl sulfate.
Source: From Ref. 24.

irritation were similar as in acute irritation, but repetitive mild irritation may evoke epidermal hyperplasia with minimal inflammatory infiltration (50).

Many surfactants including SLS disrupt the skin barrier function resulting in increased TEWL (59,60), and increased blood flow, clinically visible as erythema (61). Leveque et al. (62) suggested that an increase in TEWL did not necessarily imply the alteration of SC- and SLS-induced dry skin could hardly be interpreted in terms of lipid removal (63). A disruption of the secondary and tertiary structure of keratin proteins may expose new water-binding sites resulting in SC hydration, and the most likely explanation of SLS-induced increase in TEWL lies in the hyperhydration of SC and a possible disorganization of lipid bilayers (29). Forslind (64) proposed a domain mosaic model of skin barrier. SC lipids are not randomly distributed, but are organized in domains. Lipids with very long chain lengths are segregated in gel, impermeable to water, and separated by grain borders populated by lipids with short chain lengths, which are in fluid phase, permeable to water. Surfactants including SLS infiltrate the fluid phase permeable to water increasing the width of grain borders, and increase TEWL.

Torma et al. (65) showed that the expression of transglutaminase 1 exhibited a twofold increase after 24 hours in the SLS-exposed

TABLE 25.3
ESCD Guideline on Clinical Scoring of Subacute/Cumulative SLS Irritant Reactions

Score	Qualification	Description
0	Negative	No reaction
1/2	Doubtful	Very weak erythema and/or shiny surface[a]
1	Weak	Weak erythema, diffuse or spotty, slight scaling, and/or slight roughness[b]
2	Moderate	Moderate degree of erythema, scaling, roughness and/or weak edema and/or fine fissures
3	Strong	Marked degree of erythema, scaling, roughness, edema, fissures and/or presence of papules, and/or erosions and/or vesicles
4	Very strong/caustic	Similar to 3, with necrotic areas

[a]The term "shiny surface" is used for those minimal reactions that can only be discerned when evaluated in skimming light as a "shiny area."
[b]The term "roughness" is used for reactions that can be felt as rough or dry, sometimes preceded or followed by visible changes of the surface contour, in contrast to "scaling," which is accompanied by visible small flakes.
Abbreviations: ESCD, European Society of Contact Dermatitis; SLS, sodium lauryl sulfate.
Source: From Ref. 24.

TABLE 25.4
Noninvasive Bioengineering Techniques Used in the Evaluation of Cutaneous Irritation

Technique	Measured skin function	Informed obtained
Evaporimeter	Transepidermal water loss	Positive dose–response relationship for skin response to SLS. Most sensitive method for SLS-induced irritation
Laser-Doppler flowmeter	Blood flow	Positive relationship between applied dose of SLS and blood flows. Wide fluctuations in response to SLS due to spotty erythema
Ultrasound	Skin thickness	No preconditioning is necessary. Good relation to SLS concentrations, but minimal correlation with erythema or epidermal damage.
Impedance, conductance, capacitance	Skin hydration	Correlation with epidermal damage, but intraindividual variation is so high, this method is unhelpful.
Colorimeter	Skin colors	Positive correlation between changes in the a* color coordinates and doses of SLS, but not with epidermal damage.

Abbreviation: SLS, sodium lauryl sulfate.

skin. Profilaggrin was decreased after six hours. Later (4–7 days), the expression in SLS-exposed areas was >50% above than control areas. An increased and altered immunofluorescence pattern of involucrin, transglutaminase 1, and filaggrin was also found. At six hours post-SLS exposure, the mRNA expression of kallikreun-7 (KLK-7) and kallikrein-5 (KLK-5) was decreased by 50% and 75%, respectively. Thereafter, the expression pattern of KLK-7 and KLK-5 was normalized.

Noninvasive Bioengineering Techniques Assessing SLS Reaction

Several noninvasive bioengineering methods to quantify and obtain information that is not detectable clinically have been developed (Table 25.4) (66). Measurement of TEWL as a technique to evaluate skin barrier function is widely used (67,68). When attempting to quantify irritant patch-test reactions by electrical conductance measurement, the intraindividual variation in the results was so high that the method was found unhelpful for this purpose (69). A positive relationship was found between dose of SLS and blood flow values recorded by LD (70,71). However, wide fluctuations in laser Doppler blood flow values in response to SLS patches were found due to spotty erythema (46). The skin color is expressed in a three-dimensional coordinate system: a* (from green to red), b* (from blue to yellow), and L* (from black to white) values (72). Color a* coordinates have been demonstrated to correlate well with visual scoring of erythema in inflammatory reactions caused by soap or SLS (68,73,74). Ultrasound examination has the advantage that no preconditioning of the subjects is necessary before measurement. Ultrasound A-scan has been found suitable for quantification of patch-test reaction (75,76), and also a promising method of quantification of SLS-induced inflammatory response, being consistently more sensitive than measurement of skin color (70), and Seidenari and di Nardo (77) demonstrated that B-scanning evaluation showed a good correlation with TEWL in assessing superficial skin damage induced by SLS.

In a comparison among evaporimetry, LD, ultrasound A-scan, and measurement of skin color, evaporimetry was found to be the best suited method for evaluation of SLS-induced skin damage (68,69). Lee et al. (78) also observed that measurement of erythema index using Dermaspectrometer was less sensitive than TEWL measurement. However, Wilhelm et al. (68) suggested that although TEWL measurement may be an accurate and sensitive method in evaluating skin irritation, color reflectance measurement may be a helpful complimentary tool for clinician, because

of its convenience. Serup (79) suggested that measurement of TEWL is sensitive and useful in the study of corrosive irritants, such as SLS, especially in the induction phase of irritant reaction, but does not have direct clinical relevance, and the results need to be backed up with other relevant measures. Fluhr et al. (80) suggested that, regarding the time-dependent effect, a positive discrimination was seen for TEWL, measuring the barrier function, and the perfusion parameter LD. The discriminatory ability of TEWL was superior to that of LD. However, when evaluating SLS patch testing by bioengineering methods, TEWL measurement appears more suitable to evaluate skin reaction to SLS concentration <1.0%, whereas LD is more appropriate to evaluate pronounced skin reaction (SLS concentration ≥1%) (81).

Tupker et al. (82) found that the time course of TEWL after a 24-h SLS patch test varied between different subjects. Using SLS in varying concentrations, Serup and Staberg (76) found a delayed response only for reactions clinically scored as 1+, but not for more intense reactions, indicating that the kinetics of the response may depend on the severity of the reaction (81).

Recovery of SLS Reaction

Wilhelm et al. (83) studied the skin function during healing phase after single 24-h patch application of 0.5% SLS solution. Erythema was most increased directly after patch removal with a slow gradual decrease, but not completely resolved even 18 days after treatment. SC hydration evaluated by capacitance measurements did not return to baseline values before 17 days after surfactant exposure. The repair of the SC barrier function as indicated by TEWL measurement was completed 14 days after exposure. Freeman and Maibach (46) described augmented irritant response to repeated application of 2% SLS solution on the clinically improved ICD site, and suggested that although skin may appear to be morphologically normal, it may not be functionally normal. Lee et al. (21) suggested that complete recovery of skin function after acute reaction induced by 1% SLS solution was achieved approximately four weeks later. Choi et al. (84) demonstrated that skin reactivity of chronically irritated sites with SLS solution showed hyperreactivity compared with normal skin even 10 weeks after chronic irritation, and suggested that chronically irritated skin required a longer recovery time than acutely irritated skin.

Comparison of SLS Reaction with Noncorrosive Irritants

Irritants could be divided into two types: corrosive and noncorrosive irritants (79). Corrosive irritants induce impairment of skin barrier function even when in provoked weak or subclincal reaction. Corrosive irritants have shown linear dose–response curve. However, noncorrosive irritants that cause low degree of irritation do not induce barrier disruption, and noncorrosive irritants may show linear dose–response at lower concentrations and have a tendency to make a plateau at higher concentrations. SLS has been considered as the typical corrosive irritant, and nonanoic acid (NAA) is an example of noncorrosive irritant. There are many reports comparing the skin responses between SLS and NAA; there are clinical morphology, histopathologic changes, and changes in skin function measured by noninvasive bioengineering techniques.

Reiche et al. (85) observed the clinical morphology of SLS and NAA patch-site reactions and showed that erythema decreased with time for all concentrations of NAA and at higher concentrations of SLS. Surface changes increased with time for SLS patch sites and at higher concentrations of NAA. Lindberg et al. (86) studied the differential effects of SLS and NAA on the expression of CD1a and intercellular adhesion molecule 1 (ICAM-1) in human epidermis. ICAM-1 reactivity could not be detected in epidermis on the site of 20% and 80% of NAA solution, and there was a decrease in CD1a+ cells after 80% NAA application. However, SLS induced ICAM-1 expression on keratinocytes, and the effects on the number of CD1a+ cells were minimal. Forsey et al. (87) compared the effects of NAA and SLS on the LCs and keratinocytes of clinically normal skin in patients with chronic ICD. SLS induced keratinocyte proliferation after 48 hours of exposure; however, NAA decreased keratinocyte proliferation after 24 hours of exposure, but this returned to basal levels after 48 hours. SLS induced keratinocyte apoptosis after 24 and 48 hours of exposure; however, NAA induced epidermal cell apoptosis after only 6 hours of exposure. SLS had no effect on LC number, and no CD1a+ apoptotic cells were seen after exposure to SLS. NAA dramatically decreased LC number after 24 and 48 hours of exposure, which was accompanied by basal redistribution. Most significantly, NAA induced apoptosis in over half of LCs present after 24 and 48 hours of exposure. Boxman et al. (88) observed immunoreactive HSP27 in the upper cell layers of the epidermis after exposure to the higher (2%) concentration of SLS. However, HSP27 nuclear immunoreactivity was observed in the skin exposed to the lowest concentration of NAA tested (2.5%).

Seidenari (89) compared the irritant reactions induced by NAA and SLS using 20-MHz B-scan. A clear decrease in flexibility of the epidermis echo at 24 hours was visible at SLS patch-test sites, whereas at patch sites with NAA, there was a trend toward an increase in values of hyper-reflecting pixels. Fullerton et al. (90) studied the skin irritation typing and grading using laser Doppler perfusion imaging. For SLS, both mean perfusion and area were found to increase from day 2 to 3. The values decreased on day 5. The NAA reactions had a more rapid onset, peaked at 24 hours (day 2) and then gradually declined at 48 and 96 hours. We applied the SLS and NAA solutions on the volar forearm skin for 24 hours and measured TEWL and erythema indices to compare the different features of irritant reactions between corrosive and noncorrosive irritation. In our study of TEWL measurements, SLS solutions caused higher TEWL than NAA, and the slope of SLS curve was steeper than that of NAA curve in relation to the concentration of SLS and NAA solutions. There was a tendency for the TEWL to make a plateau at the higher NAA concentrations. However, both SLS and NAA solutions showed very similar pattern of erythema indices. In the study of the time course of TEWL and erythema indices, TEWL returned to baseline values after three weeks in areas patch tested with 50% NAA. But TEWL values did not recover baseline values until three weeks in the corresponding areas tested with 5% SLS. However, erythema index curve of 5% SLS and 50% NAA showed quite similar pattern (91). Benzalkonium chloride (BKC), another typical noncorrosive irritant, showed much less damage to the skin barrier function compared with the concentration of SLS, while they showed a similar degree of erythema. The slope of BKC was between those of SLS curve and NAA curve in relation to the concentration of SLS, NAA, and BKC solutions (92).

HOST-RELATED FACTORS

There are many host-related factors in cutaneous irritation: those that are considered as skin disease and those that represent variations from normal skin predisposed to irritation (Table 25.5).

Age

Increased susceptibility to SLS in young females compared with elderly females was reported, when assessed by visual scoring and TEWL, and the increase in TEWL values was found to be more persistent in the older group (93,94). These findings imply less reaction to an irritant stimulus but a prolonged healing period in older people. There is no significant influence on skin susceptibility between the 18 and 50 years of age (95), but significantly reduced irritant reactivity in more than 55 years age group compared with various younger age groups (96).

Sex

Hand eczema occurs more frequently among women than men. However, many investigators have found no sex correlation in skin susceptibility (47,97–99). Reactivity to SLS at day 1 increased in the menstrual cycle compared with days 9–11, when tested on opposite arms in healthy women (100). Since no cyclical variation was found in baseline TEWL, the increased reactivity of the skin at day 1 in the menstrual cycle probably reflects an increased inflammatory reactivity, rather than changes in the barrier function. Robinson (101) reported that the male subjects responded more rapidly, and there was a significant increase in response of the male subjects compared with female subjects.

Anatomic Region

Variation in skin responses within the same individual to identical irritant patch tests may be considerable. Van der Valk and Maibach (102) studied the differences in sensitivity of volar surface of the forearm to SLS and demonstrated that the potential for irritation increases from the wrist to the cubital fossa, and Panisset et al. (103) showed that TEWL values next to the wrist were found to be greater than the values on the other sites of volar forearm. Cua et al. (93) reported that the thigh had the highest reactivity and the palm the lowest. Henry et al. (104) studied the regional variability to 1% SLS using corneosurfametry bioassay and found that the dorsal hand and volar forearm were the least reactive, the neck, forehead, back, and dorsal foot the most reactive sites. Dahl et al. (105) found that, for simultaneous Al-patch testing with SLS, the corresponding sites on the right and the left sides were scored identically in only 53% of cases. Using large Finn chambers (12 mm), 84% of SLS patches showed identical visual score when tested simultaneously on right and left arms (69). Rogiers (106) suggested that measurement of TEWL should be carried out on identical anatomic sites for all subjects involved, and the volar forearm is a good measurement site and corresponding places on the right and left forearm exhibit the same TEWL.

TABLE 25.5
Host-related Factors in Cutaneous Irritation

Age
Sex
Anatomic region
Race and skin color
Skin hydration
Sensitive skin
Hyperirritable skin
Skin disease (atopic dermatitis, hand eczema, seborrheic dermatitis)

Race and Skin Color

Bjornberg et al. (107) reported that fair skin and blue eyes showed the high intensity of the inflammatory response to a mechanical irritant. When skin color was assessed by a tristimulus colorimeter, an association between light reflection (L*) from the skin surface and susceptibility to SLS was found (95). By determination of minimal erythema dose (MED) in Caucasians, the cutaneous sensitivity to ultraviolet (UV) light and to seven different chemical irritants was found to correlate positively, whereas skin phototype based on complexion and history of sunburn proved less reliable (108). McFadden et al. (109) found no significant differences in irritation thresholds to SLS among six skin phototypes. In contrast to these reports, an inclination to increased susceptibility to SLS in black and Hispanic skin types as compared with white skin types was found when evaluated by measurement of TEWL (45,67). There were more complex reports concerning the SLS susceptibility between Caucasian and Asian population. There was an increased cumulative irritation response in Japanese subjects *versus* Caucasians to various chemicals (110). Foy et al. (111) demonstrated a greater acute irritant response in Japanese women compared with Caucasian women; however, cumulative irritation did not show significant increase in Japanese compared with Caucasians. Chinese displayed similar response profile in acute irritation test; however, they showed a slower and less-severe response in the cumulative irritation test compared with Caucasian or Japanese subjects (101). Robinson et al. (112) failed to find significant differences in skin reactivity to SLS between Caucasians and Asians. However, there was a consistent trend toward increased reactivity, that is, reduced time to respond, observed in the Asian subjects *versus* Caucasian subjects (96). Tanning may influence the susceptibility to irritants. A diminished reaction to SLS after UVB exposure was reported (113).

Skin Hydration

In repetitive exposure to SLS, higher susceptibility was reported in dry skin than in clinically normal skin in eczematous subjects and controls (82). Comparing winter and summer skin, decreased skin hydration was found in winter, when a higher reactivity to SLS was also found (26). Low outdoor temperature and low relative humidity in the winter lead to decreased ability of the SC to retain water (114). Thus, these studies indicate that

a decreased hydration state of the skin may be associated with impaired barrier function and increased skin susceptibility. In contrast, Lammintausta et al. (115) found no relationship between clinically dry skin and the response to repeated SLS exposure.

Sensitive Skin

Frosch and Kligman (116) reported a significant correlation between the skin response to particular irritants in healthy volunteers and patients with skin diseases. Murahata et al. (117) suggested a relationship between skin susceptibility to detergents and high baseline TEWL, and a highly significant correlation between baseline TEWL and TEWL after a single or repeated exposure to SLS was reported (19,99,100). However, other studies reported an absent or poor correlation between baseline TEWL and TEWL after SLS exposure (45,46,118,119).

Sensitive skin is a skin type having higher reactivity than normal skin and developing exaggerated reactions when exposed to external factors (120). The stinging test using lactic acid has been widely used for the selection of sensitive skin. However, this test is based on self-perceived assessment and lacks objectivity. Seidenari et al. (121) demonstrated a decrease of baseline capacitance values indicating the tendency to barrier impairment, and they suggested that dehydration can represent a basis for subjective sensations after exposure to water and soap. Lammintausta et al. (107) demonstrated reactivity to a 24-h SLS patch test using LD in stingers compared with nonstingers. Simion et al. (122) also showed the correlations between self-perceived sensory responses to cleansing products and TEWL and colorimeter a* values in stingers. However, other studies did not show correlation between self-assessed skin sensitivity or skin reactivity to chemosensory stimuli and skin reaction to SLS irritation (96,112,123). We performed the lactic acid sting test, dimethyl sulfoxide (DMSO) test, and SLS patch tests in 55 Koreans and found no significant differences in the skin responses of these tests between sensitive and nonsensitive skin (124).

Loeffler et al. (125) propose the new classification of skin irritancy. People with sensitive skin are only individuals who stated their skin as sensitive. There is no possibility to prove the statement with objective methods. If individuals react repeatedly to a skin test with sensation induced by chemical irritants, such as lactic acid, they are identified as a stinger. If individuals do have an increased skin susceptibility to irritation caused by chemical irritants, which objectively be measured using bioengineering methods, they are identified as individuals with an irritable skin.

Irritable or Hyperirritable Skin (Excited Skin Syndrome)

Mitchell (126) introduced the term *angry back* to describe the phenomenon of a single strong positive patch-test reaction creating a back, which is hyper-reactive to other patch-test applications. The excited skin syndrome was illustrated experimentally in guinea pigs, and increased susceptibility to an ointment containing 1% SLS was observed in animals stressed by inflammatory reactions in the neck area (127). Bruynzeel et al. (128) attempted to use SLS patches as markers of hyperirritability. Agner (129) observed no increased skin reactivity to SLS in patients with chronic or healed eczema compared with controls, while hand eczema patients with acute eczema showed increased skin reactivity to SLS compared with controls. Shahidullah et al. (130) reported increased TEWL values in the clinically normal skin of patients with eczema. But there was no significant difference in baseline TEWL values between patients with eczema and controls (129,131).

Skin Diseases (Atopic Dermatitis, Hand Eczema, Seborrheic Dermatitis)

There is a marked abnormality in barrier function on the skin of patients with atopic dermatitis (AD), and high levels of sphingomyelin deacylase were demonstrated in the lesional and nonlesional skin of patients with AD leading to decrease of ceramide and abnormality in barrier function (132). Di Nardo et al. (133) suggested that SC ceramide content may determine a proclivity to SLS-induced ICD. There are many reports of increased baseline TEWL in clinically normal skin of patients with AD (82,134–138). Agner (135) showed that the response to SLS was statistically significantly increased in atopics compared with controls, when evaluated by visual scoring and skin thickness, but not TEWL. Nassif et al. (137) suggested that AD patients, as well as those with a history of allergic rhinitis, had lower irritant threshold than controls. It has also been demonstrated that a significantly greater response to SLS (82,129,137,139), as well as a tendency to increased skin susceptibility, is related to the degree of severity of the dermatitis (140). There were no significant differences in TEWL between individuals who were classified as atopic but without active dermatitis, individuals with rhinoconjunctivitis or atopic asthma, and healthy controls, either at the basal or at the post-SLS measurement. Enhanced skin susceptibility is only present in individuals with active dermatitis (141). Basketter et al. (142) also could not find significant differences in skin reactions to SLS in the normal skin of AD compared with control group.

Baseline TEWL in patients with localized, inactive, or healed eczema were not higher than in controls (15,129). Agner (129) observed no increased skin reactivity to SLS in patients with chronic or healed eczema compared with controls, whereas hand eczema patients with acute eczema showed increased skin reactivity to SLS compared with controls.

There were several reports that patient with seborrheic dermatitis could be easily irritated to some chemicals, including SLS (139,143). Tollesson and Frithz (144) observed increased TEWL and abnormality in essential fatty acids in infantile seborrheic dermatitis, and TEWL were normalized by applying the borage oil containing gamma-linoleic acid.

CONCLUSION

It is clear that SLS to date does not allow unanimity on all points. Yet, the preponderance of the observations suggest that we are beginning to understand some of the parameters, such as purity, dose, patch, anatomic site, single *versus* multiple applications, occluded *versus* open application, that influence diverse response of the skin irritation.

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26 Water: Is it an irritant?

Tsen-Fang Tsai

An irritant is defined as any agent, physical or chemical, which is capable of producing cell damage. Everything can be an irritant if applied for sufficient time and in sufficient concentration. Water, being the most abundant element of the skin, is usually regarded as banal and gentle. Thus, tepid water bath is usually recommended for the skin care of infant and atopic dermatitis (1). However, the irritancy of water is beyond doubt. (2) All nature evolves from water. However, as man evolved from water and became adapted to the earthy environment, the protection from water became one of the chief functions of the skin, which is the major protective organ of human beings. Except in the fetus, protected by vernix caseosa, prolonged soaking in water is incompatible with human life.

Irritant contact dermatitis is the hallmark of an irritant reaction. It has been traditionally classified into an acute and chronic type. Strong irritants will induce a clinical reaction in a single application, whereas with less potent irritants the response may be delayed and subclinical, requiring repeated or prolonged application (3). However, not all irritant reactions manifest as dermatitis. Water, being an unconventional irritant, may irritate the skin in a way other than dermatitis. Fingertip dermatitis, or wear and tear dermatitis, is the best example of cumulative irritant reaction. In this condition, hands are chronically irritated by a variety of insults, especially water. The involved skin is hardened and fissured, but typical signs of dermatitis or inflammation, such as erythema, swelling, or scaling are often lacking in the early stage. People who deal with wet work, such as hair dressers, hospital cleaners, cannery workers, bartenders, and hydrotherapists (4), are especially at risk (5). Sensory irritation, such as pruritus (6), pain (7), or skin tightness (8) may also occur after water exposure in susceptible patients or in normal hosts. Substance P (9) and VIP (10), respectively, have been implicated in their pathogenesis. Recently, tumor necrosis factor-alpha polymorphism was found to be responsible for this sensory irritation (11). Another water-induced condition is aquagenic urticaria (12), in which impurity and osmolarity of water may be important. Water as solvent for putative epidermal antigen has been proposed for its pathogenesis (13).

Occlusive patch test is the "gold standard" for the study of contact dermatitis and the irritancy of water under occlusion has likewise attracted most clinical attention. Prolonged warm water immersion under occlusive shoes was considered to be the culprit of tropical-immersion-foot (14). This is a condition of painful swollen feet first noticed in soldiers during the Vietnam wars. However, it is under dispute whether it is water itself or occlusion that produces the irritation (15,16). Another condition is juvenile plantar dermatosis in which children, mostly atopic, present with dry, glazed, and fissured forefeet. Repeated wet-to-dry process in conjunction with friction was incriminated as the main cause.

Occlusive dressing has long been used as an effective adjuvant therapy for diverse conditions, such as keloid (17), periungual verrucae (18), and psoriasis (19). Occlusion has been demonstrated to modify reactive events in Langerhans cells, and has profound effect on cytokine production (20). Occlusion can be achieved with either plastic dressing, silicone, or by water-soaked patches. In diseased skin, occlusion can improve skin barriers (21); however, normal skin will show typical signs of inflammation, such as vasodilation, perivenular lymphocytic infiltration, edema, mast cells degradation, and proliferation of fibroblasts after occlusion for up to two weeks (22). Agner and Serup (23) studied skin reactions after closed patch tests and 6 of 20 participants had a grade 1 clinical response to water after occlusion for 24 hours. The irritation of water under occlusion can result from water per se or from retention of sweat, which is far more irritative than water per se (24). However, a state of anhidrosis will result after prolonged occlusion (25,26).

A normal water gradient is required for a healthy skin. The outermost layer of stratum corneum contains 10–30% water, whereas the viable epidermis contains roughly 70% water. In the stratum corneum, topically applied water exerts mechanical stress on individual corneocytes, resulting in an alteration of barrier function. Treatment with distilled water results in swelling of stratum corneum cells and formation of massive water inclusions between adjacent cell layers. Corneocytes near the live–dead transition zone can swell nearly to double their thickness (27). In the viable epidermis, the control of water passage is more complex. Water can slowly permeate the lipid bilayer by simple diffusion. In addition, some specialized cell membranes show higher water permeability. Water channel proteins, aquaporins, mediate the efficient movement of water across the membrane. Skin bears abundant water channel aquaporin 3, important in the maintenance of cell volume (28).

Water, as an irritant, exerts its damaging effect on the skin through different mechanisms. Skin occlusion will induce a change in the water gradient, and an adaptation of skin physiology ensures accordingly. The normal desquamation process is highly dependent on the water gradient of the stratum corneum. Increased water content of the stratum corneum will dilute the enzymes and change the pH value important for the corneodesmolysis (29). As a result, in macerated skin, the stratum corneum shows retentional hyperkeratosis and is shed in large sheets. Water may also inactivate type 1 transglutaminase and result in a special condition called self-healing collodion baby (30). Increased water content in the stratum corneum will also have a negative feedback response

on the formation of natural moisturizing factors (NMFs) through the deactivation of keratohyalin granules degradation. Keratohyalin granules are known to be the main source of NMFs. The skin surface becomes excessively dry after the removal of occlusion. This drying effect of water is best demonstrated in wet packing for management of exudative lesions.

The importance of water as a primary irritant was demonstrated by Willis in 1973. Clinical and histologic observations of skin occluded for 72–144 hours revealed intense subacute dermatitis (31). In 1997, Ramsing and Agner have also induced experimental irritation by sodium lauryl sulfate in 21 healthy volunteers; one hand was exposed to water for 15 minutes twice daily for two weeks, whereas the other hand served as control. Water did not significantly influence transepidermal water loss, but caused a significant increase in skin blood flow, as evaluated by laser Doppler flowmetry. Clinical evaluation did not show any difference of dryness or scaling in this study (32). Without occlusion, the irritancy of water by itself is questionable in this model. However, it is impossible to clearly separate the effects of occlusion and water. The effect of occlusion must be conduction to the skin through water as a medium under physiologic condition. And although erythema alone does not equate to irritancy, temperature-stimulated erythema has been observed to augment pre-existing irritation (33). Thus, water may also exert its irritancy through its other nonchemical nature. The temperature dependency of irritation has been well recognized (34,35). Besides, hydration changes the optics of the skin, and increases the penetration and absorption of the ultraviolet light. Photobleaching of the melanin is also more prominent in dampened hairs and swimmers (36,37).

Persistent hydration of the skin surface also changes the ecologic environment and supports the overgrowth of pathologic organisms on the skin (38–41). Diaper rashes and pitted keratolysis are the best examples. Dermatophytosis complex of the toewebs is likely affected. Occlusion alone may clear the periungual verrucae, and spread the mucosal-type human papillomavirus, that is, condylomata acuminata, to the extragenital areas. Extraction of water-soluble substances, or NMFs, from skin is another mechanism. NMFs are a group of water-extractable substances, including sodium pyrrolidone carboxylic acid, sodium calcium lactate, amino acids, urea, and a sugar–protein complex. These substances can bind three to four times their own weight of water (42–44). The presence of water in the stratum corneum relies on an intercellular bilayer membrane that encloses the NMFs as in an envelope (45). Since water is the main plasticizing factor of the horny layer, the water content of the stratum corneum decreases when the NMFs are reduced, and superficial cracks might develop. The amino acid contents in senile skin are decreased (46). Showering, bathing, and hand washing removes these water extractable substances (47) and a delay in the replenishment of NMFs in aging skin may further aggravate this situation. It is for these reasons that frequent or prolonged bathing and showering, even without the use of soaps, is discouraged for the care of dry and senile skin (48).

Water may also interfere with electrolyte homeostasis and cause skin wrinkling. Water diffuses into the porous skin of the hands and soles via its many sweat ducts. Altered epidermal electrolyte homeostasis may cause changes in membrane stability of the surrounding dense network of nerve fibers and trigger increased vasomotor firing with subsequent vasoconstriction. Vasoconstriction, through loss of volume, leads to negative digit pulp pressure resulting in a downward pull on the overlying skin, which wrinkles as it is distorted. Impairment in this process may result in transient reactive papulotranslucent acrokeratoderma (49), also called aquagenic keratoderma (50) and aquagenic syringeal acrokeratoderma (51,52). It is especially common in patients with cystic fibrosis, and has been reported to occur after amikacin (53) or tobramycin treatment (54). Bedsides immersion-wrinkling test is used as a test of autonomic digital nerve function, which is impaired in diabetes mellitus and trauma (55).

The importance of skin surface acidity was only unveiled recently after a long dispute (56). This acidic milieu is vital for both the integrity of barrier function and for the regulation of skin flora (57,58). The skin surface pH has also been found to be predictor for the development of irritant contact dermatitis (59). The irritancy of water can theoretically also result from its neutral pH of 7.0, which is alkaline compared with skin surface pH of between 4.2 and 6.0. The origin of this skin surface pH has remained enigmatic and urocanic acid is likely the key factor in the maintenance of this acid mantle (60). The neutralization capacity of lesional skin in hand eczema has been shown to be defective (61). The change in skin surface pH has been shown in atopic dermatitis, ichthyosis, diabetes mellitus, and patients on dialysis.

Water is a universal solvent. The trace elements in the thermal water are the corner stone of the alleged beneficial effect of crenotherapy. On the contrary, the hardness of water may sometimes contribute to the irritancy of water (62) and atopic dermatitis (63,64). Hypotonicity of pure water, and change of water pressure gradient across the stratum corneum, which may trigger the release of cytokines, may also play a role in the irritancy of water. Specific osmotic sensitive receptor, such as TRPV4, may also be involved (65,66). The same receptor may also be activated by heat (67), low pH, and citrate (68). Hydration of the stratum corneum also facilitates the penetration of foreign substances, and contributes to the development of allergic and irritant contact dermatitis. This is best exemplified in occupational contact dermatitis involving wet work (5). Occlusive dressing therapy and wet wrapping therapy involve the same principle to enhance the therapeutic effects of topical corticosteroids (69).

Water is the most important element of the human body. The control of water passage is a highly regulated but poorly studied process. In the skin, it was previously considered to be a passive process controlled by the “dead” stratum corneum. But recent studies have revealed the importance of aquaporin, TRPV4, hyaluronic acid, and its receptor. To maintain this water homeostasis, a relatively dry and impermeable skin is highly desirable. Any change in this water gradient will bring about major changes in skin physiology. Water is a ubiquitous irritant and a well-known solvent, and exerts its irritancy through different mechanisms. It is difficult to clearly separate the effect of water itself and the trace elements contained within. The irritancy of water is controlled by the quality and quantity of water as well as by individual susceptibility, including genetic predisposition and concomitant diseases, especially atopic dermatitis. But even contact with pure water will produce physiologic changes of the skin, and these changes might be involved in some pathologic processes. Everything can be an irritant, including water.

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27 In vivo human transfer of topical bioactive drugs among individuals: Estradiol and testosterone

Kristine B. Zitelli and Howard I. Maibach

INTRODUCTION

Transdermal hormone application provides an alternative route of medication delivery. The comparatively decreased systemic exposure reduces many side effects associated with first pass metabolism, gastrointestinal absorption, and peak plasma drug concentrations. Positive clinical benefits of transdermal estradiol (1–10) and testosterone (11–15) are validated. Transdermal dosing is available in patch, gel, emulsion, and spray preparations. While patches may provide a protective covering, other formulations are directly applied to the skin, and thus may increase the risk for interpersonal transfer through hormone residue. This risk is attributed to a mass balance effect of the topical dose, allowing excess hormone to remain on the skin during a typical application period of 24 hours or more (16).

Unintentional transfer between a dosed individual and his or her interpersonal contacts is documented (17–19). Although transdermal delivery of estradiol and testosterone is clinically relevant for the treated individual, unintentional interpersonal transfer can cause hormone imbalance and adverse effects. These imbalances may increase adverse cardiac event risk in men; cause hyperandrogenism, hirsutism, acne, voice coarsening, clitoris hypertrophy, and male-pattern baldness in women; and precocious puberty, virilization, and premature epiphyseal plate closure in children (20–28). Such adverse events have gained notice in the public eye and have led to U.S. Food and Drug Administration (FDA) issued black box warnings on transdermal hormone products.

This chapter reviews the literature regarding in vivo transdermal estradiol and testosterone transfer in humans. We further explore the potential ramifications of interpersonal transfer as well as the need for optimized testing systems and topical hormone delivery methods.

METHODS

We conducted a literature review from 1950 to 2011, using the following key words: transdermal hormone transfer, absorption, estradiol, and testosterone for data related to transdermal hormone transfer in man. Unpublished data regarding transdermal product approval and product updates were reviewed on the FDA website. Public opinion regarding transdermal products was collected from Internet news sources.

RESULTS

In vivo human transfer studies of topical estradiol and testosterone are reported in Table 27.1 (28). Positive transfer is documented with both topical estradiol (29,30) and testosterone (31). Wester et al. (29) conducted an in vivo bioavailability transfer study between six healthy, postmenopausal women dosed with a 0.16 g [^{14}C]-estradiol gel formulation (5 mg [^{14}C]-estradiol radioactivity per 8.5 g of gel) and six healthy men or women. Attempted transfer was by 10 rubbing strokes and 15 minutes of direct ventral forearm contact, occurring 1 hour after hormone application. A protective sleeve was placed over the dosed site and a ^{14}C-assay was performed on skin washings, recovered 24 hours after dosing, to examine the transfer potential. Estradiol transfer occurred as indicated by a 2.3 ± 2.0% wash recovery and a 4.1 ± 3.6% sleeve recovery in the naïve, nondosed recipients. Importantly, the majority of the dose remained on the skin surface during the 24-hour dosing period and was therefore available for transfer/absorption (29).

In emulsion studies, Taylor and Gutierrez (30) performed an open-label, nonrandomized clinical trial to investigate transfer between 14 postmenopausal women dosed with 1.74 g transdermal estradiol emulsion (Estrasorb® containing 2.5 mg of estradiol/g) and 14 male partners. Attempted transfer was by vigorous skin-to-skin rubbing of 2 minutes, occurring 2 and 8 hours after estradiol application to each leg (8.7 mg total), and was measured by serum concentration changes of estradiol, estrone, and estrone sulfate in all participants. The average serum estradiol concentration in men increased from 17.0 ± 4.3 pg/mL before contact to 21.0 ± 4.4 pg/mL on day 2 after attempted transfer; signifying a 25% increase from baseline. The mean area under the curve $(AUC)_{0-24}$ from day 2 to baseline was 1.25 ($P < 0.0001$) for estradiol, 1.35 ($P < 0.018$) for estrone, and 1.16 ($P < 0.021$) for estrone sulfate indicating that significant transfer occurred (30).

In testosterone studies, Rolf et al. (31) conducted a single-center, open, randomized study involving 12 healthy male volunteers to determine what percentage of a mean 11.0 ± 1.7 mg dose of 2.5% testosterone gel applied to the abdomen and ventral forearm would remain on the skin after 10 and 30 minutes and after 1, 2, 4, and 8 hours. After 10 minutes, 60.2% (6.8 ± 1.8 mg) of the applied testosterone dose could be recovered and 8 hours later, approximately 50% (5.0 ± 2.0 mg). Notably, washing the skin with water reduced the percent recovered. The study also evaluated the potential for transfer by rubbing the dosed left forearm (11.7 ± 2.2 mg to each area) with the nondosed right forearm for 5 minutes after a 30-minute end application. Transfer occurred as 3.1 ± 1.8% of the applied testosterone dose was recovered from the nondosed forearm (31).

TABLE 27.1

In Vivo Human Transfer Studies of Topical Estradiol and Testosterone

References	Wester et al. (29)	Taylor and Gutierrez (30)	Rolf et al. (31)	ZumBrunnen et al. (32)	Schumacher et al. (33)
n	6	14	12	48	20
Dosed individual	Postmenopausal, healthy women	Postmenopausal women	Healthy men: left forearm	Postmenopausal, healthy women	Postmenopausal, healthy women
Naïve transfer recipients	Healthy men and women	Male partners	Right forearm	Postmenopausal women	Healthy men
Transdermal Product					
Hormone	Estradiol	Estradiol	Testosterone	Estradiol	Estradiol
Formulation	Gel (Estrasorb®)	Topical emulsion (Estrasorb®)	Gel 2.5%	Gel 0.06% (EstroGel®)	Transdermal spray (Evamist®)
Active ingredient**	[14C]-estradiol, [4-14C]-NEC-127 estradiol (specific activity 54.1 mCi/mmol)	Estradiol	Testosterone	Estradiol	Estradiol
Inactive ingredients**	Gel formulation	Water Ethanol Polysorbate 80 Soybean oil	Purified water Carbomer 980 Ethanol 67.0% Isopropyl myristate Sodium hydroxide	Purified water Alcohol Carbomer 934P Triethanolamine	Alcohol Octisalate
Transdermal application site					
Location	Forearm	Leg	Left forearm	Posterior arm	Forearm
Daily dose (total)	0.16 g	8.7 mg	23.4 ± 4.4 mg	1.25-g	4.59-mg (3 × 90 µL sprays)
Applied area	100 cm^2 (5.0 × 20 cm^2)	"Each leg"	2 × 1 80 cm^2 Area	"Maximal area" of posterior arm	"Three nonoverlapping sites" on each inner forearm
Amount applied/area	0.0016 g/cm^2	NA*	11.7 ± 2.2 mg	NA*	NA*
No. of daily exposures	1	2	1	1	1
Total no. of days	1	2	1	14 (×2 treatment periods)	18
Skin-to-skin contact					
Time after application (hr)	1	2, 8	0.5	1	1
Transfer area/site	Ventral forearm	Forearm	Right forearm	Posterior arm	Inner forearm
Vigorous/rubbing contact (min)	10 Strokes	2	5	3	0
Constant Contact/no movement (min)	15	0	0	12	5
Outcomes measured					
	2.3 ± 2.0% Estradiol recovered	Average serum [estradiol]	3.1 ±1.8% of applied	Postcontact serum estradiol (127.52 ± 66.60),	90% CI for ratio of post-contact
	from skin washing	increased from	testosterone dose	estrone (529.00 ± 225.27), and estrone sulfate	to precontact mean AUC (0–24)
	4.1 ± 3.6% estradiol recovered	17.0 ± 4.3 pg/mL before	was recovered from	(11.44 ± 4.29) did not increase significantly from	of 1.00–1.07; entirely contained
	from protective sleeve	contact to 21.0 ± 4.4 pg/mL	the non-dosed site	baseline serum estradiol (126.57 ± 61.79),	within a predetermined 90% CI
		after contact; 25% increase		estrone (510.46 ± 262.69), and estrone	of 0.80–1.25
		from baseline		sulfate (11.45 ± 4.01)	
Conclusion					
	Transfer to naive recipients occurred	**Significant transfer occurred**	**Transfer occurred**	**Significant transfer did not occur**	**Significant transfer did not occur**

*(NA): data Not Available; ** from manufacturer product information. *Abbreviations*: AUC, area under the curve; CI, confidence interval.
Source: Adapted from Ref. 28.

While measurable transfer is documented above, other trials have yielded different results. In a single-center, randomized, open-label, crossover, multiple-dose estradiol gel study, ZumBrunnen et al. (32) evaluated transfer potential between 48 healthy, postmenopausal women dosed with 1.25 g estradiol gel 0.06% (EstroGel®) and nontreated postmenopausal women. Attempted transfer was by 3 minutes of posterior arm rubbing and an additional 12 minutes of direct skin-to-skin contact, occurring 1 hour after application. AUC_{0-24} data indicated that postcontact serum concentrations (estradiol 127.52 ± 66.60; estrone 529.00 ± 225.27; estrone sulfate 11.44 ± 4.29) did not significantly increase from baseline (estradiol 126.57 ± 61.79; estrone 510.46 ± 262.69; estrone sulfate 11.45 ± 4.01), thus significant transfer did not occur (32).

In spray studies, Schumacher et al. (33) conducted a single-center, open-label study to evaluate transfer between 20 postmenopausal women dosed with 4.59 mg transdermal estradiol spray (Evamist®) and 20 healthy men. Attempted transfer was by 5 minutes of forearm contact without movement, occurring 1 hour after application, and was measured by precontact and postcontact serum estradiol levels in the men. The AUC_{0-24} was calculated to evaluate serum estradiol concentration of the men at 4, 8, 12, 16, 20, and 24 hours after the attempted transfer. In this trial, significant transfer did not occur as determined by a 90% confidence interval (CI) for the ratio of postcontact mean AUC_{0-24} (556.5) to precontact mean AUC_{0-24} (538.0) of 1.00–1.07, which was entirely contained within a predetermined 90% CI equivalence range of 0.80–1.25 (33).

In addition to data published in the medical literature, excerpts of in vivo transfer studies (i.e., EstroGel®, Estrasorb®, Evamist®, AndroGel®, and Testim®) are available on the FDA website (34–39).

DISCUSSION

Transdermal hormone transfer from dosed individual to naïve interpersonal contact can occur (29–31,37–39) and may cause clinically significant hormone imbalance and adverse events (20–28). Wester and Maibach (40) reported that equal-strength topical testosterone application to both an adult and newborn rhesus monkey increased systemic availability in the newborn by 2.7 times that of the adult. Likewise, transfer from parent to child is of concern (41–47).

In May 2009, the FDA issued a black box warning on the testosterone 1% gel formulations, AndroGel® and Testim®, after the reports of adverse effects in exposed children (48). The warning occurred after eight cases of secondary testosterone exposure in children, 9 months to 5 years of age, with resulting precocious puberty and/or pronounced virilization (41–48).

Public multimedia forums (e.g., newspapers and Internet sites) have additionally raised concerns with the safety of transdermal hormone products. In July 2010, *The New York Times* (49) reported that Evamist® was linked to breast development in children. On the same day, a *Los Angeles Times* blog reported that menopausal women using Evamist® should avoid contact with children and pets (50). Similarly, *HealthDay* (51), *MedPage Today* (52), and the *Plain Dealer* (53) reported this concern. The accessibility of medical information and popularity of Internet news sources allows such information to travel fast among readers and warrants a response from manufacturers.

An FDA Podcast acknowledged the eight postmarketing cases of unintended exposure of children, 3–5 years of age, to Evamist® as well as in two spayed female dogs and offered reassurance with recommendations for safe patient use (54). An important point is made that it is not feasible (i.e., likely not ethical) to conduct scientific trials of transdermal hormone transfer specifically from adults to children. Since this is the case, it is necessary to further evaluate and optimize the research designs of transdermal hormone products.

Although published data indicates positive transdermal transfer from dosed individuals to nondosed interpersonal contacts for some hormone products, the studies are difficult to compare due to variations in methods and outcomes measured. Future experiments may consider standardization of contact methods and duration of exposures based on realistic and exaggerated endpoints (28). It is difficult to compare trial outcomes because some studies attempted transfer by vigorous contact and rubbing (30,31) and concluded that transfer occurred; whereas others employed direct contact without movement (33) and concluded that transfer did not occur. Realistically, interpersonal contact may not be so straightforward.

In postmarketing cases of transdermal hormone transfer from parent to child, it is unlikely that the adverse effects were caused by transfer methods employed in the in vivo trials (e.g., direct contact without movement). Transfer more likely occurred through daily interactions, such as hugging, playing, cuddling, or even sleeping next to each other. For this reason, transfer studies based on exaggerated rubbing force and contact times may be more capable of reflecting real-life exposures (28). Furthermore, clothing transfer studies have indicated positive transfer potential (29,55,56), and thus may be useful in consideration of daily interpersonal interactions.

The controlled in vivo experiments in humans require further quantification. The excipient properties of the vehicle likely affect absorption. Even so, two of the above trials reported different outcomes with the same estradiol gel and similar attempted transfer methods. Wester et al. (29) observed a positive estradiol gel transfer via ^{14}C assay while ZumBrunnen et al. (32), via baseline changes in estradiol, estrone, and estrone sulfate concentrations, did not. Therefore, the endpoints that appear most accurate may enable quantification of hormone change after transfer through measurement of a radioisotope (^{14}C) or stable isotope as measured by mass spectrometry (28). Transdermal transfer is difficult to measure by changes in serum hormone concentrations due to interference from endogenous hormones. This is consistent with Wester and Maibach (57), suggesting that serum concentrations of an applied compound in vivo are extremely low following topical application and may only be detected by tracer methodology. Any hormone concentration changes from baseline may be too small to measure and may explain the negative studies. Furthermore, diurnal variations in serum testosterone (58–64) and estradiol (61,62) occur. Accordingly, the most accurate detection assay would be one that does not interfere with endogenous hormone concentrations (28).

The phenomenon of transdermal transfer is not unique to topical hormones. Belsey et al. (63) examined the potential for dermal exposure to pesticide residue after workers re-enter an area where foliage had been previously sprayed. The group performed spin coating of five pesticides (2,4-dichlorophenoxyacetic acid,

acetochlor, atrazine, chlorpyrifos, monocrotophos) onto different disk platforms that were subsequently air dried overnight. Loading levels were at 1000 μg/cm^2 (24 μL of pesticide, spun at 650–700 rpm for 18 s then 850–900 rpm for 60 s) and 100 μg/cm^2 (5.3 μL of 50 mg/mL pesticide mixed with 4.7 μL acetonitrile, at 1500 rpm for 18 s then 2350 rpm for 60 s). The in vitro experiment involved applying the pesticide residue-coated platforms against porcine abdominal skin for 24 hours. Data indicated that acetochlor (liquid) and monocrotophos (highly hygroscopic) remained in liquid form and penetrated the skin faster than their aqueous solutions. The behavior of the chemicals was consistent with the notion that compounds with lower melting points permeate the skin more rapidly. Notably, each pesticide resulted in residues with different properties; substances with solid residues generally resulted in very low permeation compared with aqueous solutions (63). The authors note that, like the transdermal hormone describe above, permeation of the pesticides through the skin may depend on other unmeasured variables, such as perspiration of the exposed individual, wet platform surface (e.g., foliage), or high humidity. Like transdermal hormone transfer, studies on dermal exposure during re-entry may benefit from experiments performed under realistic conditions.

Frictional properties of the skin and regional variations may also affect transfer. The friction coefficient interprets skin differences on various anatomic locations and between individuals and monitors skin changes after topical compound use (64,65). The friction coefficient of the skin may be increased with hydration or decreased with a drying agent (64). Transfer potential is also complicated by *application* site of the dosed individual and *contact* site of the naïve recipient (28). Regional variation of percutaneous absorption is highest in the genital region, followed by the face, trunk, arms, and legs (66,67). Penetration at the forearm may underestimate absorption at all body sites (66), which is worth mentioning because many in vivo trials attempt transfer via forearm contact. By understanding the mechanical state of the skin, it may be possible to devise better transdermal application systems that focus on the site of application, character of the vehicle, as well as differences in age, gender, race, and anatomic sites of the dosed individual (28).

In theory, any transdermal hormone residue that is not absorbed may become available for transfer. Therefore, it is important to understand the details of percutaneous absorption and expand our knowledge regarding transfer potential. The *10 Steps to Percutaneous Absorption*, defined by Wester and Maibach in 1983 (57), may serve as a foundation to develop transdermal products with low probability of interpersonal transfer.

The continued development of optimal transdermal hormone products is warranted. Xing et al. (68) studied the transport properties of estradiol absorption in each layer of the skin (i.e., stratum corneum, epidermis, and dermis); providing a basis to guide appropriate drug administration. An enhanced understanding of the transport properties of transdermal hormones in each skin layer may reveal intrinsic differences in product absorption, and thus potential for interpersonal transfer. Other topical treatment options, such as a hydroalcoholic 2.5% testosterone gel that is removed by washing 10 minutes after application (69), are being compared to barrier delivery methods (i.e., testosterone patch) to evaluate differences in biological effect and hopefully, decrease potential for transfer. Alternative to conventional delivery methods (e.g., patch or gel), a combination of electroporation and iontophoresis may result in enhanced transdermal hormone permeation (calcitonin and parathyroid hormone in this experiment) (70). Further studies are warranted to determine if increased absorption via advanced transdermal delivery methods can also decrease interpersonal transfer by decreasing residual hormone on the skin surface.

CONCLUSION

Transdermal hormone transfer from dosed individual to naïve interpersonal contact can occur and may cause clinically significant hormone imbalance and adverse events. In response, the FDA recently issued a black box warning on topical testosterone products and the media continues to raise concerns with transdermal hormone transfer from adult to child. The phenomenon of transdermal transfer is not, however, unique to topical estradiol and testosterone. Unintentional transfer probably depends on many variables that are challenging to assess under research trial conditions. Optimization of in vivo studies is necessary to reduce transdermal transfer by interpreting data based on realistic interpersonal interactions and standardized outcomes, developing superior topical formulations and advanced transdermal delivery methods, and attaining an overall greater understanding of percutaneous absorption of topical agents on human skin.

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28 Pigmentation changes as a result of arsenic exposure

Nikolay V. Matveev and Molly L. Kile

INTRODUCTION

Arsenic, the 20th most abundant element in the earth's crust, is present in trace levels in all soil, rock, water, and air. Arsenic compounds can exist in both inorganic and organic forms and are classified according to their valence states: elemental 0, arsenite (trivalent, 3+), and arsenate (pentavalent, 5+).

Inorganic arsenic is released into the atmosphere and natural waters from weathering and dissolution of arsenic-containing minerals, volcanic activity, microbial activity, and anthropogenic activities.

Arsenic can be present at high concentrations in nonferrous ores, including copper, zinc, and gold, and is a common contaminant of coal. Biologic activity can transform inorganic arsenic into organic forms. Seafood can contain high concentrations of organic arsenic but these species are considered to be nontoxic.

The biologic effect of arsenic will largely depend on the arsenic species, dose, and duration of exposure. The International Agency for Research on Cancer has determined that there are sufficient human data to classify inorganic arsenic as a known human carcinogen (1).

SOURCES OF EXPOSURE TO ARSENIC

Humans can be exposed to arsenic from environmental, medicinal, and occupational sources, as well as from intentional or accidental poisoning.

Environmental Exposure

Consumption of arsenic-contaminated drinking water is the primary route of exposure for most individuals. In many countries, aquifers pass through arsenic-rich geologic strata resulting in groundwater with elevated arsenic levels.

Most notably affected by arsenic-contaminated groundwater are Bangladesh, West Bengal, India, Inner Mongolia, China, Mexico, and Chile. Increasing reliance on groundwater for drinking water in South East Asia has also led to an increased prevalence of arsenicosis in Pakistan, Vietnam, Laos, and Cambodia.

Surface waters can also become contaminated from anthropogenic sources. For instance, arsenic concentrations in acid mine drainage can reach very high concentrations. Arsenic concentrations are also high in the geothermal waters in Kamchatka, Japan, Alaska, and California (2).

Bottled water can contain arsenic if the source water comes from a contaminated aquifer. For instance, Armenian mineral water "Jermuk" was found to contain arsenic at concentrations ranging up to 600 μg/L and has been the subject of several recalls by the United States Food and Drug Administration (3). This popular mineral water has been widely available in the Soviet Union for decades; now it is widely sold at least in Armenia and Russia.

Food grown in contaminated soil or irrigated with contaminated water can accumulate arsenic. Total dietary studies have shown that rice, cereal grains, and products made from these grains, including breakfast cereals, crackers, and rice milk are the primary sources of inorganic arsenic in the diet (4–8,80). Seaweed and seafood contain high concentrations of organic arsenic species, including arsenocholine and arsenobetaine, commonly called "fish arsenic," which are considered to be relatively nontoxic (9). Seaweed-based dietary supplements can contain elevated levels of organic arsenic and consumers who take large doses of these kinds of supplements can be exposed to high levels of organic arsenic (10,11) Thus, an individual's dietary exposure will depend on their food habits and the food's agricultural origin.

Medicinal Exposure

Arsenic has a long history in medical and veterinarian medicines. In 1786, Thomas Fowler developed a solution of 1% potassium arsenite, which now bears his name. Fowler's solution was touted as a general tonic for ailments ranging from jealousy to cancer and was most likely the first effective treatment for leukemia (12).

Arsenic's antimicrobial properties were exploited by Paul Ehrlich in 1907, who synthesized Salvarsan (3,3-diamino-4,4-dihydroxyarsenobenzene dihydrochloride) (13). Both substances were phased out of use by the mid-1900s due to the development of more effective treatments and concerns about carcinogenic risks.

Currently, arsenic trioxide (As_2O_3) is used to treat acute promyelocytic leukemia (14) and as a treatment for trypanosomiasis (15). It can also be an ingredient in folk remedies, particularly of Asian origin (16). A survey conducted in Boston of the commonly available South Asian Ayurvedic and homeopathic medicines found arsenic concentrations ranging from 37 to 8130 μg/g, which if taken regularly could result in arsenic toxicity (17).

Occupational Exposure

In 2009, the annual world production of arsenic was estimated to be 54,400 tons with 75% of the production occurring in China, Chile, and Peru (18). The United States is the world's leading consumer of arsenic mainly by the wood preservative industry,

production of agricultural chemicals, glass manufacturing, and the production of gallium-arsenide (GaAs), indium–arsenide (InAs), and indium–gallium–arsenide (InGaAs) for the semiconductor industry. Voluntary bans on using arsenic-based wood preservatives have led to an overall decrease in arsenic production but the popularity of "smart phone" technology has seen a surge in the production of high-purity arsenic for the semiconductor industry.

In addition to the extraction and refining of arsenic ores, occupational exposures most often occur in copper smelting, mining, glass manufacturing, and semiconductor industries.

Some coal deposits can also have elevated arsenic concentrations depending on localized geologic factors. Coal miners in the Guizhou Province of China are at particular risk because coal deposits in this region contain very high concentrations of arsenic. In this region, it is common for coal to be burned inside the home for cooking and crop drying purposes, resulting in chronic arsenic exposure from contaminated indoor air and food (19).

Woodworkers can be exposed to inorganic arsenic from wood treated with chromium, copper, and arsenic (CCA), a common preservative. These workers can have dermal exposure from improperly cured wood or from breathing sawdust (20,21).

Inorganic arsenic is also added as an active ingredient in some commercially prepared insecticides, herbicides, and rat poisons. Inorganic arsenic-based herbicides, including lead arsenate, calcium arsenate, and sodium arsenate, once widely used in agriculture, have largely been banned in Western countries out of concern for worker safety. However, herbicides containing organic arsenicals, such as monosodium methanearsonate (MSMA), disodium monomethylarsonate (DSMA), calcium acid methanearsonate (CAMA), and cacodylic acid have taken their place and are currently used in cotton agriculture and in lawn care.

Intentional or Accidental Poisoning

In the Middle Ages, arsenic was a well-known poisoning agent because it was tasteless, odorless, fatal at very low doses, and incurable. Its popularity as an agent of assassination declined only in the nineteenth century when forensic medicine developed reliable detection methods. In the modern era, accidental poisoning or attempted suicide is the most common reason for acute arsenic poisoning.

A notable example of accidental poisoning occurred in Manchester, England, in the early 1900s, which afflicted 6000 beer drinkers and resulted in 70 fatalities (22). The source of the arsenic contamination was traced to the glucose, or brewer's sugar, made from starch that had been hydrolyzed with sulfuric acid, which was heavily contaminated with arsenic. Analysis of the beer revealed that a gallon of beer contained anywhere from 65 to 195 mg of arsenic (23).

Another mass arsenic poisoning affected infants of the western part of Japan in 1955 (mostly in Okayama Prefecture), because of arsenic-contaminated milk powder. More than 100 bottle-fed babies died from acute toxicity and others developed abnormal skin pigmentation, diarrhea, and fevers. 0-up studies of these bottle-fed infants who were exposed for up to four months indicated that the exposed infants had a higher incidence of neurologic disorders, skin disorders, and increased mortality from skin cancer, liver cancer, and leukemia (24).

Burning wood that has been treated with chromated copper arsenic preservative (CCA-treated wood) can also lead to unintentional exposures through dermal exposure to soot and ash, and indoor air pollution. This was documented in a family who used scraps of CCA-treated plywood to heat their home during the winter. All eight members of the family experienced arsenic-associated health problems, including pruritic dermatitis, pneumonia, severe diarrhea, peripheral neuropathy, hair loss, and reddening and thickening of the palms and soles (25).

Burning coal in homes can also lead to unintentional exposure. In the Guizhou Province of China, coal, which is traditionally burned in open pits inside the home for heat and cooking purposes, can contain up to 35,000 ppm of arsenic, and can produce arsenic concentrations in indoor air up to 400 mg/m^3 resulting in inhalation exposure and also contamination of food supplies stored in the home or cured over the coal fires (19).

ABSORPTION, METABOLISM, AND MECHANISMS OF TOXICITY

Approximately 80–90% of a single oral dose of inorganic arsenite (As3) or inorganic arsenate (As5) is absorbed from the gastrointestinal tract (26,27). Airborne arsenic can be readily absorbed through the lung. The bioavailability and subsequent toxicity will likely depend upon the physicochemical properties of the arsenic compound (28).

Percutaneous absorption of soluble arsenic compounds is also possible but dermal absorption rates are very low and estimated to be 2.0–6.4% of the applied dose (29). Once absorbed, arsenic is metabolized through a series of oxidative, reduction, and methylation reactions. This process begins when arsenate is reduced to arsenite in the bloodstream with glutathione (GSH) acting as the electron donor. S-Adenosyl methionine then transfers a methyl group to arsenite that is subsequently oxidized to form monomethylarsonic acid (MMA5). MMA5 can undergo an additional reduction to form methylarsonous acid (MMA3) that is methylated to form dimethylarsinic acid (DMA5), which can be further reduced to dimethylarsinous acid (DMA3) (30). All these species have been detected in urine collected from arsenic-exposed individuals (31,32).

Unlike other mammals, humans excrete a large percentage of MMA. Population surveys indicate that the average distribution of urinary arsenic species in humans is 10–30% inorganic As, 10–20% MMA, and 60–70% DMA (30,33). The relative proportion of urinary arsenic species is frequently used in epidemiologic studies to characterize an individual's arsenic methylation capacity.

Traditionally, methylation of inorganic arsenic has been considered a detoxification process because the mono- and dimethyl arsenic species are more readily excreted in urine than inorganic arsenic (34). However, experimental studies indicate that the trivalent methylated arsenic intermediates (MMA3 and DMA3) may be more toxic than arsenite (As3) or any of the pentavalent arsenic species (35). Epidemiologic studies show that low secondary arsenic methylation capacity as defined by a higher ratio of MMA-to-DMA, or a higher proportion of MMA in urine, are associated with the severity of arsenic-related skin lesions (36). It has also been shown that the risk of skin lesions is only associated with the percentage of urinary MMA where the odds of arsenic-induced

skin lesions increased with $\log_{10}$ percentage of MMA [adjusted odds ratio = 1.56, 95% confidence interval (CI): 1.15, 2.12] but not $\log_{10}$ percentage of inorganic arsenic or $\log_{10}$ percentage of DMA (37).

Thus, factors that influence arsenic metabolism, such as age, gender, nutritional status, or genetics could explain why some individuals are more susceptible to arsenic toxicity than others.

Arsenic toxicity is a product of the concentration and duration of exposure of each arsenic species at the target site. All organ systems are susceptible to arsenic. The mechanism of arsenic toxicity is poorly understood. At a biochemical level, pentavalent arsenic can replace phosphate in chemical reactions and trivalent arsenic has a high affinity for thiol groups, which can disrupt cellular enzymes and uncouple oxidative phosphorylation (38). In addition, trivalent arsenic species generate reactive oxygen species. For instance, DMA3 reacts with molecular oxygen to form a dimethylarsinic radical and a superoxide anion. This superoxide anion then generates hydrogen peroxide, which can form a hydroxyl radical that can damage DNA (39,40).

There is also a growing evidence that inorganic arsenic influences epigenetic mechanisms, including DNA methylation, histone regulation, and microRNA expression (41). For instance, experimental and epidemiologic studies have shown that arsenic exposure is associated with increased methylation in the tumor suppressor genes p15, p16, p53, *RASSF1A*, *PRSS3*, and death-associated protein kinase (DAPK). Increased methylation within the promoter region silences gene expression, and aberrant DNA methylation is associated with many chronic diseases, including cancer.

DERMAL EFFECTS

There is sufficient human evidence to classify arsenic as a known human carcinogen and that exposure to arsenic increases the risk of skin cancers (1). The first report of arsenic-induced skin cancer was published in 1885 by Dr. White, of Harvard Medical School, who described ulcerative lesions on the palms in a patient who took Fowler's solution for a number of years to treat psoriasis; the lesions appeared to be epitheliomas (42). It was Sir Jonathan Hutchinson who examined Dr White's patient later in England and first suggested that arsenic could be a cause of skin cancer (43).

The carcinogenic effect of Fowler's solution was later proved in rats whose skins were brushed with a 1.8% solution of potassium arsenite (44). Epidemiologic studies have demonstrated that the odds of developing skin cancer, including Bowen's disease, squamous cell carcinoma, and basal cell carcinoma, are significantly associated with drinking arsenic-contaminated water (45).

Chronic arsenic exposure induces a series of characteristic skin changes proceeding from hyperpigmentation to hyperkeratosis (46). Significant associations have been observed between hyperpigmentation and palmar/plantar hyperkeratosis and risk of skin cancers (47).

A unique characteristic of arsenic-induced Bowen's disease (carcinoma in situ) is that they are confined to sun-protected regions of the body, unlike sun-induced Bowen's disease. This difference could be explained by an interaction between arsenic and ultraviolet (UV) light. In vitro experiments have observed that combined exposure to UVB irradiation and arsenic increases the number of apoptotic cells resulting in an inhibitory effect on cellular proliferation (47,48).

Skin is a target site for arsenic toxicity because trivalent arsenic has a high affinity for sulfhydryl groups, which are highly concentrated in keratin (47). In human skin cell lines comprised of keratinocytes, melanocytes, or dendritic cells, arsenic demonstrated both cytotoxic and genotoxic activities (49). Although the mode of arsenic carcinogenicity has not been fully established, it is thought that arsenite (As3) binds to thiol groups and inhibits DNA repair, whereas arsenate (As5) replaces phosphates in DNA causing chromosomal aberrations and deletion mutations. Arsenic also alters DNA methylation and suppresses keratinocyte differentiation (47).

BIOMARKERS OF ARSENIC EXPOSURE

Biomarkers are useful tools for evaluating exposure to environmental pollutants as they are quantitative measures of biologically relevant doses and reflect the internal dose from all exposure pathways. Arsenic can be measured in the blood, hair, nails, and urine. It is important to consider the half-life of arsenic in each of these tissues and individual exposure histories to select and interpret biomarker data. For instance, a single dose of arsenic is cleared from the bloodstream within several hours and excreted by the kidney so blood arsenic concentrations are considered a poor biomarker of past exposures. Recent advances in analytical instrumentation have led to improved detection limits and arsenic can now be accurately detected in blood. Blood arsenic levels may provide useful exposure information for individuals who have a blood test immediately after an exposure or who are chronically exposed to consistently high levels of arsenic (50).

In urine, a single dose of ingested arsenic has a half-life of approximately 26 hours (51), whereas the half-life of repeated oral exposures is approximately three days (52), making urinary arsenic measures a useful biomarker for recent exposures. Background urinary arsenic concentrations for unexposed individuals range from 0.1 to 1.0 ppm. It is important to realize that urinary arsenic measurements that only measure total arsenic, and not individual arsenic metabolites, can be confounded by seafood consumption (53,54). Therefore, if urinary arsenic is being used to determine exposure status, the patient should not eat seaweed, shellfish, or other seafood in the three days prior to the collection of the urine sample. However, if an analytic technique is being used that can separate the different arsenic metabolites, then it is possible to remove the interference from the organic arsenic contributed from seafood and get a more accurate estimate for exposure to inorganic arsenic.

Hair and toenails, on the other hand, are useful biomarkers for historical exposures. Inorganic arsenic binds to keratin and becomes isolated from metabolic activity, which would lead to its excretion in the urine. Subsequently, arsenic can accumulate in hair and nails (55). Average background arsenic concentrations in nails ranges from 0.43 to 1.08 ppm, whereas median background arsenic concentration in hair is approximately 0.5 ppm (56). In healthy chronically exposed populations, toenail arsenic concentrations are highly correlated with drinking water arsenic levels ($R2 = 0.73$) (55).

Epidemiologic studies show stronger correlations between drinking water arsenic and hair arsenic concentrations in both in healthy subjects ($R2 = 0.35$ vs. 0.57) and subjects with skin lesions ($R2 = 0.72$ vs. 0.85) compared with blood arsenic concentrations (57). Furthermore, hair and nails can be collected noninvasively

and do not require specialized handling or storage. Although arsenic will present itself quickly in the base of the hair shaft and nail bed, it takes several months to a year for arsenic to reach the distal tip of the nail where it can be collected. Thus, nails provide a useful biomarker of past exposures.

ARSENIC-INDUCED SKIN PIGMENTATION

Frequently, the earliest symptoms of chronic arsenic toxicity are pigmentation changes in the skin and the thickening of the outer horny layer of the palms and soles. Generally, it is believed that pigmentation changes occur earlier than hyperkeratosis (58) and can include a finely freckled pattern of hyperpigmentation and hypopigmentation of the skin on upper chest, arms, and legs, poetically described as "raindrops on a dusty road." More specifically, this raindrop pattern consists of less pigmented, round spots that are several millimeters in diameter on a background of diffuse hyperpigmentation (Figs. 28.1 and 28.2).

The early stages of keratosis are characterized by bilateral thickening of the palms and soles. Subsequently, multiple nontender, horny papules develop on the keratotic skin of the palms and soles, although they may also develop on the dorsum of the hands (Fig. 28.3). The papules are small, ranging from 0.2 to 1 cm in diameter, which can coalesce to form larger plaques with nodular, wart-like, or horny appearance.

According to Mazumder (59), chronic arsenic toxicity can be diagnosed from observing the presence of hyperpigmentation, keratosis, or both. Traditionally, hyperpigmentation was linked to both arsenic deposition in melanocytes and increased melanin production (60), although there also appears to be underlying toxic effects by arsenic on both melanocytes and keratinocytes. The toxic effects on keratinocytes would be relevant to the pathogenesis of hyperkeratosis (49).

Chronic arsenic exposure is associated with an increased risk of basal and squamous cell skin cancer (45,47,61), and hyperpigmentation and keratosis are considered to be premalignant conditions.

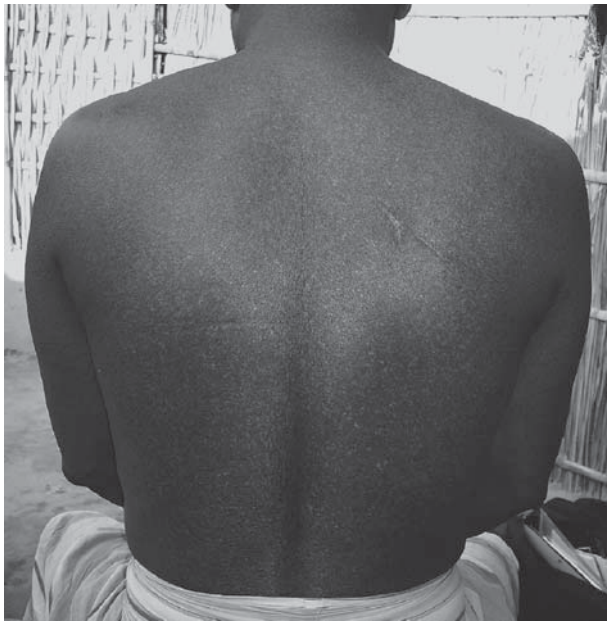

FIGURE 28.1 Characteristic raindrop pattern of hyperpigmentation associated with ingestion of arsenic-contaminated water (Pabna, Bangladesh).

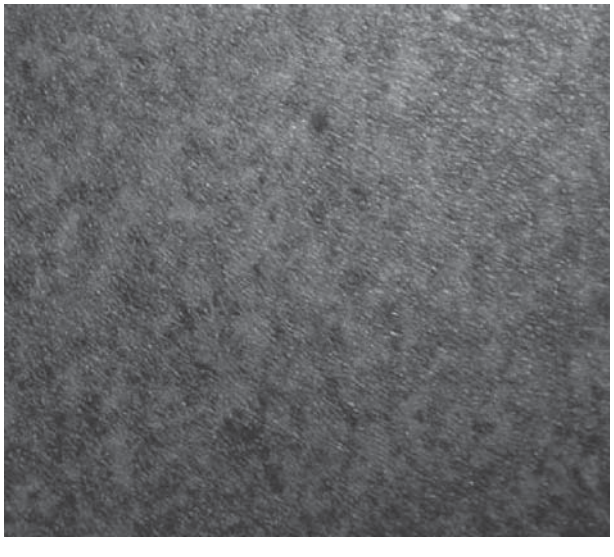

FIGURE 28.2 A closeup picture of hyperpigmented skin area (corresponds to approximately 3 × 3 cm skin area).

EPIDEMIOLOGIC EVIDENCE OF ARSENIC-INDUCED SKIN PIGMENTATION

In West Bengal, India, and Bangladesh, millions of individuals have been exposed to arsenic-contaminated drinking water from shallow tube wells. In these populations, it has been reported that arsenic-induced skin lesions appear after 5–10 years of exposure to arsenic-contaminated drinking water (62). However, arsenic-induced hyperpigmentation has been observed in children as young as 18 months in Bangladesh.

The type of skin lesion and the severity of the lesion likely depend on both the ingested dose and the duration of exposure. A population-based survey conducted in Matlab, Bangladesh, which screened 166,934 individuals above four years of age for arsenic-induced skin lesions observed a crude prevalence of 0.3% (63). This study found that 39% of the identified cases only had pigmentation changes, 5% only had keratosis, and the remaining 56% had both types of skin lesions. They also observed that men had a higher prevalence of skin lesions than women, which has been reported by other researchers (62,64–66).

The prevalence of skin lesions is related to arsenic exposure. Age-adjusted prevalence rate for skin lesions in males ranged from 18.6 per 100 skin lesion cases when drinking water arsenic concentrations were below 150 μg/L to 37.0 per 100 when drinking water arsenic concentrations were above 1000 μg/L (64).

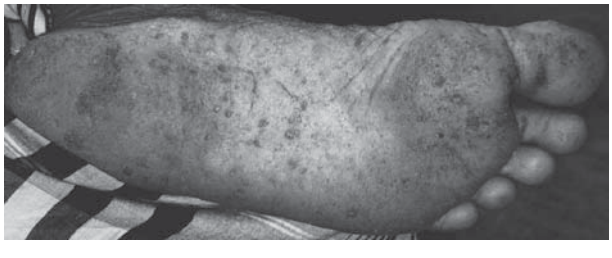

FIGURE 28.3 Plantar hyperkeratosis and pigmentation (Pabna, Bangladesh).

The analysis of the data derived from the Health Effects of Arsenic Longitudinal Study (HEALS), a longitudinal cohort study of 10,182 Bangladeshi adults initially free of skin lesions reports an increased incidence of skin lesions with increased drinking water arsenic concentrations. Compared with individuals who were drinking water that contained ≤10.0 μg As/L, the hazard ratios for incident skin lesions for different drinking water arsenic exposures (10.1–50.0, 50.1–100.0, 100.1–200.0, and ≥200.1 μg/L) were 1.17 [95% confidence interval (CI): 0.92, 1.49], 1.69 (95% CI: 1.33, 2.14), 1.97 (95% CI: 1.58, 2.46), and 2.98 (95% CI: 2.40, 3.71), respectively (67).

Another study by Mazumder et al. (51) also showed that the age-adjusted prevalence of keratosis increased with drinking water arsenic concentrations. In chronically exposed adults in West Bengal, the age-adjusted prevalence of keratosis ranged from 0.95 per 100 adults for drinking water arsenic concentrations ranging from 50 to 99 μg/L up to 9.5 per 100 adults when drinking water arsenic concentrations were above 800 μg/L. In this study, hyperpigmentation was more frequently observed. The age-adjusted prevalence rates of hyperpigmentation in Mazumder's study ranged from 2.0 per 100 adults for drinking water arsenic concentrations ranging from 50 to 99 μg/L up to 17.1 per 100 adults when drinking water arsenic concentrations were above 800 μg/L.

Epidemiologic studies in other populations exposed to arsenic-contaminated drinking water report different prevalence rates of arsenic-induced skin lesions. In Taiwan, the prevalence of hyperpigmentation and hyperkeratosis was 18.3% and 7.1%, respectively (58), whereas a survey of arsenicosis patients in North Mexico found that 12% of the patients had skin hyperpigmentation, 18% had hypopigmentation, and 11% had hyperkeratosis (68). However, as a detailed description of the observed lesions is omitted, the high prevalence of "hypopigmentation" could merely reflect terminology if the authors were referring to hypopigmented spots on hyperpigmented background instead of the more common "raindrop hyperpigmentation," which is utilized in studies originating out of South East Asia.

In cases of acute or subacute arsenic intoxications in European countries, the occurrence of hyperkeratosis, not hyperpigmentation, is more frequently reported. For instance, an observation was made in a cohort of German wine growers, who would drink a wine substitute made from pressed grapes called "Haustrunk" that had been contaminated with arsenic-based pesticides (69). Among the 163 wine growers, who consumed an estimated 3–30 mg arsenic from Haustrunk daily, 77% presented with hyperkeratosis and only 44% had hyperpigmentation.

A large meta-analysis of 143 cases of skin cancer due to arsenic exposure performed by Neubauer (70) found reports of hyperkeratosis in 116 cases (81%), whereas hyperpigmentation was only mentioned in 25 cases (17%), although direct information on the absence of hyperpigmentation was only provided for 11 cases, leaving room for speculations about the real hyperpigmentation prevalence.

Fierz (71) mentioned that among 262 cases of side effects of skin diseases treatment with inorganic arsenic, hyperkeratosis was the most frequent (40.4%), whereas hyperpigmentation was found only in five patients.

These reports on the prevalence of different types of arsenic-induced skin lesions give a general impression that in European countries the prevalence of hyperpigmentation is lower than that in Asian countries and that keratosis might be the most frequently encountered indication of arsenicosis in European populations. It is unclear what would cause a higher prevalence of keratosis in Europe and Mexico compared with Asian countries, although genetic differences in arsenic metabolism, racial specificity of melanocytes function, or confounding from sun exposure could be involved.

It can be difficult to compare prevalence rates from different studies because of differing inclusion criteria. Currently, there are no widely accepted diagnostic criteria of arsenicosis (72). Also, many studies rely upon a field diagnosis of skin lesions by a single observer, which could lead to misclassification, particularly if the skin lesion is moderate or no exposure assessment is completed. Utilizing digital photography of arsenic-induced skin lesions could provide a more objective analysis of skin lesions. Images could then be evaluated by several experienced dermatologists and could potentially reduce misclassification. The use of automated skin images analysis might also contribute to better classification (and quantification) of skin lesions, which could be especially important for longitudinal studies that are examining how skin lesions change over time.

Nutritional Influence on Skin Lesions

Nutritional status influences susceptibility to developing skin lesions. A nested prospective study in the HEALS study observed that individuals who ate a diet rich in gourd vegetables (ridge gourd, snake gourd, pumpkin, ghosala, parwar), spinach stalks, green papaya, and root vegetables (sweet potato and radish) had significantly a lower risk of developing skin lesions after six years (73). A large cross-sectional epidemiologic study in an arsenic-exposed population in Bangladesh also reported that B vitamins, folic acid, and vitamins A, C, and E significantly reduced the risk of arsenic-related skin lesions at any given arsenic exposure level (74). Also, a placebo-controlled double-blind supplementation trial demonstrated that administration of folic acid increased arsenic methylation and subsequently led to reduction of concentrations of both total blood arsenic and MMA, by 14 and 22%, respectively (75). This finding indicates that nutritional status, particularly nutrients involved in one-carbon metabolism and antioxidant defense, can modify arsenic toxicity.

DOSE–RESPONSE RELATIONSHIP

There is much uncertainty regarding the dose–response relationship between arsenic and skin lesions. This is largely due to the magnification of arsenic toxicity by dose and duration of exposure and the different methodologies employed in epidemiologic studies.

For instance, a study conducted in Mexico specified that a person must consume approximately 2 g of arsenic before developing hypopigmentation and 3 g of arsenic before developing hyperpigmentation and hyperkeratosis for 8–12 years, respectively (68).

A study in Bangladesh found that the odds of arsenic-induced skin lesions and arsenic-contaminated drinking water increased in a dose-dependent fashion. For instance, it was observed that individuals drinking water containing 8.1–40.0, 40.1–91.0, 91.1–175.0, and 175.1–864.0 μg/L had adjusted prevalence odds ratios of skin lesions of 1.91, 1.26, 3.03, and 3.71, respectively, and were 5.39 times more likely to have arsenic-induced skin lesions compared with individuals drinking water with less than 8.1 μg As/L (76).

This study is also one of the first to estimate the effect of chronic low-level arsenic exposure on the risk of developing skin lesions. Specifically, they observed that individuals consuming drinking water with 10 μg As/L had a 1.22 times higher risk of developing skin lesions compared with individuals drinking arsenic-free water. However, the relationship between drinking water arsenic concentrations and the type of skin lesion was not significant although hyperpigmentation is considered to be an earlier sign of arsenicosis compared with hyperkeratosis (76).

TREATMENT

Despite mankind's long history with arsenic, no treatment exists that can reverse chronic arsenic toxicity. Therefore, all efforts should focus on identifying and eliminating the source of arsenic exposure.

In acute poisoning, chelation therapy is frequently employed to facilitate excretion of arsenic in urine. In the United States, two chelating agents are available: 2,3-dimercapto-1-propanol [dimercaprol or British Anti-Lewsite (BAL)] and meso-2,3-dimercaptosuccinic acid (Succimer). A third chelating agent, sodium 2,3-dimercapto-1-propane sulfonate (DMPS) is available in Germany. BAL is administered through intramuscular injections and has a narrow therapeutic:toxic ratio and side effects are frequently reported. Succimer and DMPS are administered orally or intravenously and are more widely tolerated. All three chelating agents rapidly increase urinary excretion of arsenic, although no controlled studies have been performed to determine the most effectual therapy.

In cases of chronic arsenic exposure, it is unclear whether chelation therapy offers any benefit. One small placebo-controlled study conducted in West Bengal found that patients given arsenic-free water and DMPS four times a day for one week, and repeated in after three, five, and seven weeks, had significantly increased urinary excretion of arsenic compared with the patients given arsenic-free water and a placebo. While the group that received DMPS had more improvement, both groups showed significant improvement in their clinical conditions indicating the positive effect of removing arsenic exposure and improved nutrition (77). Another study that used Succimer as the chelating agent did not observe any difference in the clinical improvement compared with the placebo group (78). Therefore, it appears that DMPS is a superior chelation therapy for chronic arsenicosis, although providing clean drinking water and improving nutrition are also effective treatments for chronic arsenicosis and are recommended due to their lack of side effects.

It is believed that antioxidants, such as vitamin C, vitamin E, and selenium, may impede carcinogenic effects of arsenic. It was shown that supplementation with vitamin E and organoselenium, either alone or in combination, slightly improved the status of arsenic-induced skin lesion, although the observed improvement was similar to the improvement observed in the placebo group (79). It was demonstrated that administration of folic acid increased arsenic methylation and subsequently led to reduction of concentrations of both total blood arsenic and MMA, by 14% and 22%, respectively (75).

This finding supports earlier research that suggests that for chronic arsenicosis, removing the source of arsenic exposure and eating a well-balanced diet is important.

CONCLUSIONS

In Asian countries, changes in skin pigmentation are the first visible signs of chronic arsenic toxicity. This can include diffuse hyperpigmentation with scattered hypopigmentation. This condition can occur prior to, or simultaneously with, keratosis of the palms and soles. In European countries, keratosis might be the first indication of chronic arsenic toxicity. Considering that these arsenic-induced skin lesions are earliest signs of arsenic poisoning, and also a frequent predecessor of skin cancer, it is important to diagnose these skin lesions as early as possible to identify populations at risk and sources of arsenic exposure.

In addition, collaborative efforts should be undertaken to develop an international consensus on diagnosis of arsenic hyperpigmentation. Changes in skin pigmentation can be objectively documented with digital cameras and also quantitatively measured with portable colorimetric devices, which would help to standardize field examinations of the patients with risk of arsenicosis. The same technique could be used to determine changes in lesions as a result of treatment. Needless to say, the precise measurement of pigmentation changes can also be used to obtain more precise information in epidemiologic studies, which may additionally contribute to better understanding of arsenic toxicity mechanisms that are not fully understood.

ACKNOWLEDGMENTS

The authors appreciate the support received from Harvard School of Public Health (Dr David Christiani and Dr Stephanos Kales), and also assistance from all the collaborators of Dhaka Community Hospital and Pabna Community Clinic 2006). The researchers were partially funded by the United States National Institutes of Health (ES011622 and ES017800).

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29 Gender and pharmacokinetics*

Bobeck S. Modjtahedi, Maureen Lloyd, Nader Movassagh, and Howard I. Maibach

Advances in pharmacology have resulted in a more nuanced understanding of pharmacokinetics. The possible role of gender in drug metabolism is an area of increasing interest. Basic physiologic differences between men and women have long been appreciated to play a role in drug utilization. The Food and Drug Administration (FDA) has made a significant effort to examine gender-based differences in pharmacologic parameters (1). Some of the differences between men and women's processing of medications are the result of differences in surface area, weight, and percent body fat; however, there is growing evidence that innate differences in enzymatic activity may be at play. Gender-related differences have been observed with a large range of medications, including psychiatric medications, beta-blockers, opioids, and aspirin (2). Here we discuss several of the factors that are important in differences between the genders with regard pharmacologic response.

The absorption of medications when administrated orally is influenced by gastric factors. Women have been found to have less gastric acid (approximately 0.5 unit greater pH) as well as slower gastric emptying and intestinal transit times (2–5). Bile acid composition has also been found to vary based on gender (6). The bioavailability of medications, such as ketoconazole, which rely on gastric pH may be lower in women, and therefore there may benefit from co-administration with an acidic drink (2). The effect of slower gastric emptying in women may be mitigated by longer waits after eating before administrating medications intended for use on an empty stomach (2). Sex hormones impact gastric emptying times with menstrual and pregnancy status possibly playing a role (7).

Differences in topical absorption remain less well studied. Topical absorption is largely dictated by location and area of exposure. Factors in topical administration that may be influenced by gender include skin thickness, presence of sweat, and subcutaneous fat (7,8). Specifically hormonal status can play a significant role in skin pigmentation, status of hair, glandular activity, mucus membranes, and vascular systems—the most significant example of this may be the changes that take place during pregnancy (9). The toxicology literature provides some insights into the differences topical absorption. McCormick and Adbel-Rahman found that absorption of trichloroethylene was decreased by testosterone and that female rats had markedly more absorption than male rats (10).

Distribution is influenced by body fat (higher in women) and body size (higher in men). Plasma volume as well as protein-binding capacity also play a role in gender-based differences. The higher body fat content in women results in lipophilic drugs possessing longer duration of action (secondary to larger volumes of distribution) and conversely hydrophilic drugs having higher plasma concentrations due to smaller volumes in women (2).

The cytochrome P450 (CYP) system is principally responsible for drug metabolism and is an active area of research in gender-based differences. Table 29.1 provides the CYP enzymes responsible for the metabolism of key dermatologic drugs. Studies examining gender differences in CYP activity have demonstrated conflicting results. This may be the result of large intersubject variability, differences in study population, differences in study size, and variable effect on age. Hormonal differences between men and women differ based on the age of the subjects as well as menstrual state of female subjects and, if not accounted for, may explain some variability between studies. Bebia et al. provided a large indepth examination of differences in the major CYP groups (11). CYP1A2 had decreased activity in women, especially when factoring in smoking status of subjects. Prior studies had shown that CYP1A2 activity changed during the course of the menstrual cycle (12,13). Bebia et al. did not find CYP2C19 activity to be significantly different between the genders but did show that activity decreased with age; however, other investigators have found higher activity in Chinese female subjects than male subjects (14). Bebia et al. found that CYP2D6 was equivalent in male and female subjects, although other studies have shown significant variation between men and women showing evidence of higher activity depending on the substrate being tested (11). CYP2E1 had an age-associated increase in activity that developed later in life among female subjects, supporting the contention that age-related changes in women's hormonal status may affect this enzyme's activity (11). The authors did not find significant differences in CYP3A activity between genders. Additionally, they concluded that age appeared to increase variance rather than direction of change.

Bebia et al. found extremely large intersubject variation in CYP enzyme activity. Gender was found to be a factor in CYP1A2 activity, whereas both age and gender influenced CYP2E1 activity. CYP2C19 and 3A4 were influenced by age but not gender, whereas CYP2D6 was influenced by neither (11). A significant limitation of in vivo activity tests is that they often rely on the use of a single-tested substrate, which may not be generalizable. Ultimately, the clinical applicability of the differences in CYP enzyme activity between men and women remains to be seen and may in fact be mitigated by the large intersubject variability.

*This chapter is substantially revised and updated from Ref. 5, with permission.

Table 29.1
Metabolism of Common Dermatologic Drugs

Drug name	Drug family	Metabolism
Erythromycin	Macrolide (antibiotic)	CYP3A4 (60)
Clindamycin	Lincosamide (antibiotic)	CYP3A4 (61)
Gentamicin	Aminoglycoside	Excreted Unchanged (62)
Hydrocortisone	Glucocorticoid	CYP3A4 (60)
Betamethasone	Glucocorticoid	CYP19A (63)
Triamcinolone	Glucocorticoid	CYP3A4 (63)
Fluocinolone	Glucocorticoid	CYP3A4 (63)
Desonide	Glucocorticoid	CYP3A4 (63)
Clobetasol	Glucocorticoid	CYP3A4 (63)
Clotrimazole	Imidazole (antifungal)	CYP3A4 (63)
Miconazole	Imidazole (antifungal)	CYP3A4 (63)
Ketoconazole	Imidazole (antifungal)	CYP3A4, CYP2C19 (60)
Terbinafine	Squalene monooxygenase inhibitor	CYP2D6 (60)
Isotretinoin	Retinoid drug	CYP2B6, 3A4, 2C8, 2C8 (62)
Fluorouracil	Pyrimidine analog	CYP1A2, 2A6, 2C8 (63)
Benzoyl peroxide	Antibacterial acne treatment	Excreted by kidneys as benzoate (64)
Silver sulfadiazine	Sulfonamide	Glucuronyl transferase and *N*-α-acetyltransferase (62)

CYP3A4 system deserves particular attention given its preeminent role in drug metabolism. CYP3A is responsible for metabolizing more than 50% of the drugs currently marketed and is the most abundant member of the CYP450 family in the liver (15,16). Several investigations into hepatic CYP3A4 content and function did not show significant gender-related differences (17–19). A review by Meibohm et al. stated that the authors did not believe that any gender-related differences existed with regard to CYP3A (20). However, one review (21) described a 1.4× greater activity of CYP3A4 in women than men (22,23). An examination of CYP3A4 in human livers did find important differences between men and women (24). Wolbold et al. examined surgical liver samples and observed gender-based differences in both CYP3A4 expression and activity (based on *N*-dealkylation of verapamil) (24). They found twofold higher levels of hepatic CYP3A4 in female livers than male livers based on microsomal protein content ($P < 0.001$), and also found activity to be higher in women than in men by approximately 50% ($P < 0.01$) (24). Hunt et al. employed erythromycin *N*-demethylation in microsomes to quantify hepatic CYP3A activity in vitro (25). Women demonstrated 24% higher CYP3A activity than men in this study ($P = 0.027$) (25). Greater CYP3A activity in women has also been demonstrated for both Japanese (26) and Chinese (27) subjects.

Much of CYP3A's importance is derived from its dual presence in the intestine and the liver (15). Intestinal levels of CYP3A are of particular importance in the examination of orally administered drugs; yet the vast majority of CYP3A research focuses on the liver. Paine et al. examined the content of CYP3A4 and CYP3A5 by use of duodenal biopsies. Neither enzyme demonstrated gender-related differences (28). After restricting examination of CYP3A4 to white individuals or those that did not show CYP3A5 expression, men and women still demonstrated no difference in CYP3A4 levels (28). Although activity was not measured directly in this study, the authors noted that a strong correlation between duodenal protein content and activity has been noted in the past (29,30), implying that CYP3A4 activity would similarly not show differences between men and women (28). The applicability to investigations into the activity of isolated CYP3A in either hepatic or intestinal samples to clinical practice remains limited. Lutz et al. found that metabolic ratio of 6-beta-hydroxycortisol to cortisol in urine, an endogenous marker of CYP3A activity, was markedly increased in females to males (4,31).

Given the considerable disagreement regarding CYP3A and gender, Greenblatt and von Moltke provided an indepth review of the male-to-female clearance ratios of 38 drugs metabolized by CYP3A (32). The authors excluded drugs also transported by P-glycoprotein (Pgp) to avoid any confounding effect. The authors found considerable variability both between different drugs and among studies of the same drug (32). There was an overall statistically higher clearance of drugs studied in healthy young women compared with healthy young men (female/male average of 1.26 for parenteral drugs, 1.17 for oral drugs, $P < 0.01$). The authors concluded these differences were likely of limited clinical value. Most of the drugs studied were not weight adjusted in dosage. Women's lower average body weight would likely offset their slightly higher weight-normalized clearance among drugs given in absolute dosing regimens (32).

Pgp is a transmembrane efflux protein that plays a particularly important role in pharmacokinetics, specifically in oral and hepatobiliary clearance (33,34). Substrates metabolized by CYP3A have the tendency to act as substrates or inhibitors of Pgp (16,35). Pgp is especially important because it is expressed in a variety of tissues (36,37). Furthermore, unlike CYP3A, Pgp is recognized to be polymorphic (38). Bebawy and Chetty provide an excellent review of Pgp and gender (39). Although female sex hormones have been found to induce transported expression in animals and in vitro models, clinical reports have suggested reduced expression and function in women (39). Differences in level of Pgp expression in the liver and gut remain poorly understood with conflicting data (39). Schuetz et al. found men to have greater hepatic Pgp expression than women by roughly two orders of magnitude (40). Potter et al. (41) analyzed data made available by Lown et al. (42) and found that women had lower enterocyte Pgp content than men. However, Paine et al. (28) and Wolbold et al. (24) found no differences between the genders with regard to intestinal or hepatic Pgp expression, respectively. Steiner et al. examined MDR1 phenotype in B-type chronic lymphocytic leukemia patients (33). Far more men than women were positive for the phenotype (89% vs 48%, $P < 0.001$) (33). Table 29.2 provides a summary of the above data on CYP and transporter-related differences between genders.

Disease state may influence drug transporter status. Dickinson et al. examined transporter levels peripheral blood mononuclear cells (PBMC) of HIV-positive patients. Median expression of Pgp and MRP1 were both lower in women than in men ($P = 0.0016$ and $P = 0.018$, respectively) (43); however, no significant difference was noted in breast cancer resistance protein (BCRP) ($P = 0.395$) (43). Robertson et al. found no differences between men and women with MDR1 genotype and CYP3A activity (44). König et al. examined BCRP and multidrug resistance-associated protein (MRP) mRNA expression in normal and carcinoma pancreatic

TABLE 29.2
Sample of CYP3A and Transporter Gender-Related Differences

Author	Region	Protein investigated	Gender prevalence
Wolbold et al. (24)	Hepatic	CYP3A4 activity/ expression	Female
		Pgp expression	//
Paine et al. (28)	Intestinal	CYP3A4 expression	//
		Pgp expression	/
Schuetz et al. (40)	Hepatic	Pgp expression	Men
Lown et al. (42)*	Enterocyte	Pgp expression	/
Dickinson et al. (43)	Peripheral blood mononuclear cells (PBMC)	Pgp expression	//
		MRP1 Expression	//
		BCRP Expression	No difference
König et al. (45)**	Pancreatic tissue	BCRP Expression	//
		MRP1 Expression	//
		MRP4 Expression	//

* Data analyzed in Ref. 41
** Data analyzed in Ref. 5

tissue (45). BCRP, MRP1, and MRP4 mRNA expression did not appear to be effected by tumor stage or grading. Using the data made available by König et al., Modjtahedi et al. studied possible gender-based differences in BCRP, MRP1, and MRP4 mRNA expression. After removing observations <0.1% (one male in the BCRP group and one female in the MRP4 group), there was no statistically significant difference between the mRNA expression of the aforementioned transporters (5).

Ohno et al. investigated the expression of MRP1 mRNA in T-cell leukemia cells. There was no observed correlation between gender and MRP1 expression (46). Gutmann et al. examined the mRNA expression of BCRP along the gastrointestinal tract (47). While an earlier study observed gender differences in the distribution of animal BCRP (48), Gutmann et al. found no such variation between men and women's expression of BCRP (47). This finding emphasizes that observed gender-based differences in transporter expressions in animals are not always readily applicable to humans (21). Gutmann et al. concluded that sex hormones did not play a role in the expression of BCRP in the human intestine (47). Steroid and xenobiotic receptor (SXR), CYP3A4, and MDR1 expression did not show any significant differences between men and women in adult tissues (49).

Hormonal status may play a role in the expression of both CYP3A and Pgp; however, results have been mixed. Kim and Benet found that Pgp was inducible in vitro by sex-steroid hormones at both the protein and mRNA level (50). Growth hormone secretion patterns differ between men and women and have been noted to affect CYP3A activity (28,51). Nakamura et al. found that CYP3A4 metabolism was affected by androgen and other endogenous steroids; however, testosterone's exact role in CYP3A4 metabolism has yet to be entirely elucidated having been noted to both activate and inhibit CYP3A4 (52–54). Zhu et al. observed that CYP3A activity decreases from preovulatory to ovulatory to luteal phase ($P < 0.05$) (27). However, Wolbold et al. did not demonstrate a difference between pre- and postmenopausal women's levels of CYP3A4 protein expression (24). Furthermore, they observed that other studies have not found a difference in CYP3A4 drug metabolism based on menstrual cycle (55,56). Studies on midazolam have not demonstrated significant influence of menstrual cycle on clearance and plasma levels (7,57,57). Overall the role of menstrual and menopausal status in drug processing remains conflicting (7). The same group found that menopausal status affected prednisone but not erythromycin clearance with hormone replacement therapy not affecting either drug (58).

Gender differences are important to note with regard to renal clearance. Glomerular filtration rate is consistently higher in men, even after adjusting for body size (2,59). Those medications that are excreted unchanged in the urine get cleared more slowly in women (2,59).

Saquinavir is an important protease inhibitor used in the treatment of HIV, and serves as a useful model drug for discussion on gender-based differences in pharmacokinetics. We have provided an indepth review of this topic, which the reader may refer to for additional details (5). In short, HIV-positive women on saquinavir have frequently demonstrated higher plasma concentrations of this drug; however, the source of this difference may not be intrinsic to their gender. Possible differential effects of the HIV virus on hormonal status as well as the use of other medications may explain some of the differences observed more so than innate differences in CYP3A levels (5). This underscores the complexity of gender-based study into pharmacokinetics—other variables, such as differences in how the disease state may affect the genders, may play a role in the ultimate clinical picture.

The study of the role gender plays in pharmacokinetics has made significant strides in the past 10 years; however, much remains sub judice. Although we have an increasingly clear view of differences in enzymatic activity and transporter expression, the translation of this into clinical practice and prescribing practices remains tenuous. Going forward, additional study should focus on whether clinical outcomes can be improved by factoring in gender into prescribing medication.

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30 Dermatologic drug dosage in the elderly*

Anna Flammiger and Howard I. Maibach

INTRODUCTION

As the proportion of the world's older population continues to increase (1), the need for individualized drug dosage in this population is on the increase. This chapter discusses drug dosage and administration in elderly patients with special emphasis on drugs prescribed by dermatologists.

In practically all regions of the world the older population is growing faster than the total population (1). The fastest-growing proportion of the elderly population in many countries comprises individuals who are 80 years or older (2). The number of persons 60 years or older increased from 205 million in 1950 to 606 million in the year 2000 and is projected to reach nearly 2 billion in 2050 (1). It is projected that in 2050 more than one in every five persons throughout the world will be 60 years or older, whereas nearly one in every six will be at least 65 years old (1).

Dermatologic diseases are common among the elderly (3). According to data from the 1996–1997 National Ambulatory Medical Care Survey visits to dermatologists encompassed 4.6% of all physician visits (4). Patients in the age between 65 and 74 years had the highest number of visits with an average of 97 million visits per year (4). The three most common dermatologic diagnoses in patients 55 years or older were actinic keratosis, asteatotic dermatitis, and nonmelanoma skin cancer (4).

To improve drug dosage in the elderly factors that might influence drug pharmacokinetics and pharmacodynamics in this population are reviewed.

PHARMACOKINETICS

There are a number of physiologic changes with aging that may affect drug absorption, distribution, elimination, and metabolism (Table 30.1).

Absorption

Intestinal drug absorption may be altered by nutritional deficiencies, partial gastrectomy, and interactions with laxatives, antacids, and drugs that decrease gastric emptying (5), but it appears that there are no major alterations with age (6).

Age-related changes in the skin may impair percutaneous drug absorption (7). In the elderly the stratum corneum is drier and sebaceous gland activity is reduced. The reduced water content makes aged skin less attractive to hydrophilic compounds, such as hydrocortisone, benzoic acid, acetylsalicylic acid, and caffeine, whereas the absorption of the lipophilic compounds testosterone and estradiol is similar in young and old individuals. Compromised microcirculation may decrease the absorption capability of the aged skin.

However, no significant differences between young and old individuals have been demonstrated in the absorption of drugs from transdermal delivery systems, such as estrogen or progesterone patches for hormonal replacement therapy or fentanyl patches for chronic pain (8).

Distribution

Body composition changes in the elderly and may lead to altered drug distribution (9). Lean body mass and total body water decrease, whereas fat as a percentage of body weight increases (10,11). Consequently, the volume of distribution is decreased for hydrophilic drugs leading to higher plasma concentrations as demonstrated for alcohol (12,13). Conversely, the volume of distribution is increased for lipophilic drugs, which may result in retention and prolonged half-life (14). This has been demonstrated for hydroxyzine (Table 30.2) (15).

Elderly patients may have significantly reduced body weight (16), a major risk factor for overmedication (17).

Drugs may be bound to the plasma proteins albumin and α1-acid glycoprotein with only the free fraction being pharmacologically active (12). The concentrations of albumin and α1-acid glycoprotein may change with age (18), thus the ratio of bound to free drug may be altered (19). However, changes of more than 50% in the free fraction have been documented for only a few drugs, such as naproxen and acetylsalicylic acid (20) and mechanisms such as increased drug elimination may counterbalance the increase of the free fraction (12).

Elimination

Renal function generally declines in the elderly: Renal blood flow is decreased and tubular function is impaired reducing the kidney's ability to maintain homeostasis in stressful conditions (21). The glomerular filtration rate (GFR), calculated by the creatinine clearance, declines by around 30% between the age of 30 and 80 years (22), which can prolong the half-life of many drugs (19). Drugs may accumulate to toxic levels if dosage is not reduced (19).

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TABLE 30.1
Physiologic Changes with Aging

Pharmacokinetics	Age-related Changes	References
Absorption	Hydration of stratum corneum ↓	(7)
	Skin surface lipids ↓	
	Skin microcirculation ↓	
Distribution	Lean body mass ↓	(10,11)
	Total body water ↓	
	Body fat ↑	
Elimination	Renal blood flow ↓	(21,22)
	Tubular function ↓	
	Glomerular filtration rate ↓	
Metabolism	Liver size ↓	(44,45)
	Liver blood flow ↓	

Thus, renal function assessment is essential. Unfortunately, it is often overlooked in elderly patients (23). In one study that included 60 elderly patients with renal function impairment, 27 (45%) of the patients were receiving renally eliminated drugs in dosages that exceeded the manufacturers' recommendations (24).

Methotrexate (MTX) is mainly eliminated by the kidney (25) and its half-life may be significantly increased in patients with impaired renal function (26,27). MTX clearance may be decreased by as much as 44% in patients with a creatinine clearance of < 45 mL/min when compared with patients with a creatinine clearance of 80 mL/min (27). Patients with impaired renal function have a higher overall rate of toxicity and are at a higher risk for severe toxicities than patients with normal creatinine clearance (Table 30.2) (28). Dose reduction of MTX may be necessary in elderly patients with impaired renal function. In one study which included elderly patients with psoriasis and decreased creatinine clearance, their disease could be controlled with less than the recommended dose of MTX (29). Four patients >80 years of age were treated adequately with ≤2.5 mg weekly (29).

Like MTX, the antihistamine cetirizine is predominantly eliminated unchanged in the urine (30). In elderly patients with impaired renal function, the elimination half-life of cetirizine was significantly prolonged and the total body clearance reduced by 64% (Table 30.2) (31). It has therefore been suggested that the cetirizine dosage should be reduced by 50% in patients with impaired renal function (32).

Pregabalin is effective in reducing pain in patients with postherpetic neuralgia (33). Postherpetic neuralgia is the most common complication of herpes zoster, with the elderly being most frequently and seriously affected (34). Pregabalin clearance is reduced with age-related changes in renal function and dosage reductions, based on the creatinine clearance may be necessary (Table 30.2) (33). In patients with a creatinine clearance of >30 to ≤60 mL/min who received pregabalin 100 mg three times daily the pregabalin plasma concentration was similar to patients with a creatinine clearance >60 mL/min who received pregabalin 200 mg three times daily (35).

These studies show that assessment of renal function is important when prescribing drugs that are predominantly eliminated by the kidney. Renal function can be estimated by the assessment of creatinine clearance. One should keep in mind, however, that creatinine clearance is only a rough estimate of the glomerular filtration rate as creatinine is also secreted in small amounts by the kidney (36). The easiest way to estimate the creatinine clearance of patients is to utilize the Cockcroft and Gault equation (37), which corrects serum creatinine by age, sex, and weight:

Estimated creatinine clearance (mL/min) = 1.2 × (140 − age [years]) × weight (kg)

Serum creatinine (μmol/L) (× 0.85 for women).

TABLE 30.2
Prescribing Specific Dermatologic Drugs in the Elderly Population

Drug	Notes	References
Hydroxyzine	Prolonged half-life possible	(15)
Methotrexate	Serious potential for adverse effects with decreased renal function; screen for abnormal liver function tests, as it can cause hepatic injury	(28,57,58)
Cetirizine	Total body clearance reduced; dosage should be reduced by 50% in patients with impaired renal function	(31,32)
Pregabalin	Dosage reductions based on the creatinine clearance may be necessary	(33)
Itraconazole	Increased risk of liver damage; liver function tests should be performed in patients with pre-existing liver disease	(61–65)
Acitretin	In patients with liver disease, the dose of this drug should be reduced and liver function tests monitored closely	(69)
Systemic corticosteroids	Adverse effects such as memory loss, diabetes mellitus and depressive symptoms may occur more frequently; increased risk for peptic ulcer disease in combination with nonsteroidal anti-inflammatory drugs	(84–87)
Diphenhydramine	Increased risk of cognitive decline	(89)
Erythromycin	Strong inhibitor of CYP3A4; may lead to increased toxicity of co-administered drugs, such as benzodiazepines, calcium channel antagonists, anticoagulants, cyclosporine, and tacrolimus	(100,101)
Ciprofloxacin	Inhibits the metabolism of theophylline by CYP1A2; may result in theophylline accumulation and toxicity; may increase risk of seizures	(99)

However, creatinine clearance estimated using this equation can significantly deviate from true creatinine clearance in elderly patients (38). Due to declining muscle mass, serum creatinine might not rise significantly even if renal function is significantly impaired (22). Goldberg and Finkelstein have shown that this can lead to an overestimation of the creatinine clearance (38). In addition, patients with obesity and ascites may excrete less creatinine than is estimated with the Cockcroft and Gault equation (37). A 24-hour creatinine clearance measurement can be performed, but even with this test unreliable results are possible. For example, urine collections by patients may be incomplete (38). The clearance of exogenous substances, such as inulin, iohexol, ^{51}Cr-EDTA, ^{99m}TcDTPA, and ^{125}I-iothalamate, has been proposed as the "gold standard" for the estimation of GFR (39). However, these techniques are expensive and require administration of substances making this approach unsuitable in daily clinical practice. Serum cystatin C has been proposed as a new endogenous marker of GFR and may be a more reliable marker of GFR in the elderly than serum creatinine or creatinine clearance (40).

As a general approach, when treating patients with decreased renal function, therapeutic drug levels may be maintained either by reducing the dose or by increasing the interval between doses or both (41).

Reduced renal function may also affect drug metabolism in the liver (42). Animal studies have shown a significant downregulation of hepatic cytochrome P450 (CYP450) metabolism in chronic renal failure (43).

Metabolism

A decrease in liver size (20–40%) and a reduction in liver blood flow have been observed in the elderly (44,45). This leads to a reduced hepatic clearance of many drugs.

In the liver, transformation of lipophilic drugs to more polar products takes place (46). Phase I reactions convert the drug to a more polar metabolite and if the metabolite is sufficiently polar, it may be readily excreted at this point. However, many phase I metabolites undergo a phase II reaction, where an endogenous substrate, such as glucuronic acid is conjugated.

Phase II reactions seem to be less affected by aging than phase I reactions (47–49). However, drug metabolism in the liver is a controversial matter. In one study, CYP450 drug metabolism was reduced by around 30% after 70 years of age (49), whereas other studies found no significant age-related differences in the activity or content of human liver microsomal enzymes (50,51).

In some cases interindividual variability in enzyme activity may exceed age-related differences. Isoform CYP3A4 accounts for approximately 28% of the total human liver CYP450 content and is involved in metabolism of drugs, such as cyclosporine, dapsone, lidocaine, verapamil, and macrolides (46). Hunt et al. studied the effect of age on the activity of CYP3A and found this enzyme to be unaffected by normal aging (52). However, the enzyme showed large interindividual differences, making drug dosages difficult to determine (52).

The aging liver has a decreased capacity to recover from injury (53,54). Therefore, a history of liver disease should lead to caution in the dosage of drugs that are primarily cleared by the liver (19).

Drug-induced liver disease seems to occur more frequently in the elderly (55). For example, the incidence of developing isoniazid-induced liver damage increases (56).

Several commonly prescribed dermatologic drugs potentially cause liver damage. MTX can cause hepatic injury and older age at onset of therapy has been shown to be a risk factor (Table 30.2) (57,58). Other risk factors for hepatic fibrosis identified in patients with psoriasis who were treated with MTX include history of or current excessive alcohol consumption, abnormal liver function tests, history of liver disease, including chronic hepatitis B or C, history of inheritable liver disease, diabetes, obesity, and history of exposure to hepatotoxic drugs (59). It is therefore important to screen for abnormal liver function tests and hepatitis B and C, and to take a history of alcohol consumption (60). It is difficult to make recommendations about the need for liver biopsies for elderly patients maintained on long-term MTX as the risk of the procedure may exceed the benefit in some patients (60).

Users of itraconazole are at increased risk of liver damage, which is associated with a cholestatic pattern of injury (Table 30.2) (61–64). Although serious liver problems are rare with the use of itraconazole, liver function tests should be performed in patients with pre-existing liver disease (63,65).

Liver function tests are also recommended when prescribing fluconazole, although this antifungal preparation appears to be less toxic than itraconazole (61,65).

Severe hepatic injury with the use of acitretin appears to be a rare adverse effect of treatment with this drug (66,67). In a study by Roenigk et al., one in 83 patients treated with acitretin for psoriasis experienced moderate to severe hepatic fibrosis and no patient showed evidence of cirrhosis (68). However, in patients with liver disease, the dose of acitretin should be reduced and liver function tests monitored closely (Table 30.2) (69). Other potentially hepatotoxic drugs prescribed in dermatologic practice include agents, such as tetracycline, erythromycin, flucloxacillin, ketoconazole, azathioprine, and synthetic androgens (69).

PHARMACODYNAMICS

Receptor number and affinity, signal transduction, cellular responses, and homeostatic mechanisms may be altered in the elderly (70,71). Age-related changes have been demonstrated for adrenergic, muscarinic, and dopaminergic receptors as well as for the GABA-A–benzodiazepine complex (16). Sensitivity to certain drugs changes in the elderly. Sensitivity to benzodiazepines is increased (72), whereas the response to certain β-adrenoceptor agonists and antagonists seems to be decreasing with age (18).

Pruritus is a common skin problem in the elderly (3) and may be treated with hydroxyzine (73). Simons et al. measured changes in suppression of histamine-induced wheal and flare and suggested an enhanced suppression of H1 receptor activity by hydroxyzine in the elderly (15).

ADVERSE DRUG REACTIONS

Elderly patients are at higher risk of experiencing adverse drug reactions (74,75). However, patient-specific physiologic and functional characteristics should also be considered and may be more important than chronologic age (76).

In a study performed in the Emergency Department of the Toulouse University Hospital a significant incidence of adverse drug reactions leading to hospital admissions (8.37 per 100 admissions) was found among the elderly (77). The most important risk factors associated with adverse drug reactions were the number of drugs

being taken, self-medication, use of antithrombotics, and use of antibacterial drugs (77).

Indeed, a clear correlation exists between the number of adverse drug reactions and polypharmacy (78,79). Pilotto et al. found that in a population of more than 3000 elderly subjects, the mean number of drugs taken daily was three and an increase in the mean medication number was noted with advancing age (80).

Elderly patients with poor nutritional condition and impaired renal function are especially at risk of experiencing adverse drugs reactions (81).

Some examples of dermatologic drugs that may cause adverse drug reactions in the elderly are discussed in the following sections.

Systemic Corticosteroids

Systemic corticosteroids are particularly useful in the treatment of acute hypersensitivity diseases, connective tissue diseases, and the more common dermatoses when these are severe and widespread (82). Unfortunately, this drug class has adverse effects on many organ systems (83). The elderly may be particularly vulnerable to certain adverse effects, such as memory loss, diabetes mellitus, and depressive symptoms (Table 30.2) (84–86). Patients who received prednisolone in a dose of 5–10 mg/day for a period of at least one year experienced partial loss of explicit memory, and elderly patients may be more susceptible to memory impairment with a shorter treatment course (84). Furthermore, the risk of developing diabetes mellitus more than doubles in elderly patients who are initiated on systemic corticosteroids (85). Data from a population-based cohort of 2804 adults ≥55 years of age suggest a positive association between depressive symptoms and corticosteroid use, especially in the older subjects (86). An increased risk for peptic ulcer disease has been reported in patients who received corticosteroids in combinations with nonsteroidal anti-inflammatory drugs (NSAIDs; Table 30.2) (87). This is particularly important as patients receiving systemic corticosteroids are likely to be receiving NSAIDs as well, given that acetylsalicylic acid and other NSAIDs are among the most prescribed drugs in old age (80,87). In elderly outpatients in north-eastern Italy, NSAIDs were the third most used drug class (24.7%) after angiotensin-converting-enzyme inhibitors (38%) and diuretics (26.7%) (80). Patients on corticosteroids and NSAIDs should be closely monitored for gastrointestinal adverse events. Prescription of gastroprotective agents may improve safety in these patients (88).

Antihistamines

First-generation antihistamines bind to the H1-receptor and prevent histamine interactions with the receptor (32). However, the ability to cross the blood–brain barrier gives rise to potential central nervous system (CNS) side effects (32). Elderly patients may have a heightened risk of CNS adverse effects, such as dyskinesia, confusion, sedation, and reduced mental alertness (32). Elderly hospitalized patients ≥70 years of age, who were treated with the first-generation antihistamine diphenhydramine had an increased risk of cognitive decline compared with nonexposed patients (Table 30.2) (89). The diphenhydramine exposed group had a 70% increased risk of cognitive decline. Furthermore, diphenhydramine exposed patients were at increased risk for inattention, disorganized speech, altered level of consciousness, and behavioral disturbance. The number of reports of sedation with second- and third-generation antihistamines loratadine, cetirizine, fexofenadine, and acrivastine was low, as reported by Mann et al. (90) However, this study did not focus on the elderly. Affrime et al. (91) studied the pharmacokinetic parameters of the third-generation antihistamine desloratadine in different age groups, including elderly patients and concluded that daily administration of desloratadine 5 mg is well tolerated and no dosage adjustment is required in the elderly.

Antibiotics

Elderly patients are at increased risk of developing skin and soft tissue infections (92). Skin and soft tissue infections, urinary tract infections, and respiratory tract infections are the most common types of infections among elderly persons in long-term care facilities (93,94). Changes in skin consistency, immunosenescence, the presence of underlying skin conditions, and immobility predispose the elderly to skin and soft tissue infections (95,96). Longer stays in intensive care units, transitional units, and nursing homes increase the risk of acquiring drug-resistant strains of *Staphylococcus*, *Streptococcus*, and *Enterococcus* species among the elderly (97). Treatment guidelines recommend a penicillinase-resistant β-lactam, a first-generation cephalosporin, or clindamycin as the preferred agent for treating skin and soft tissue infections (98). However, as these guidelines were not developed specifically for the elderly population, dosage adjustments have to be considered in patients with renal or hepatic impairment (92). In addition, drug interactions should be kept in mind when selecting an antibiotic regimen. Common drug interactions with antibiotics include macrolides and fluoroquinolones (99).

Erythromycin is a strong inhibitor of CYP3A4 and may thus be responsible for toxicity of co-administered drugs by decreasing their clearance (Table 30.2) (100). Substrates of CYP3A4 include benzodiazepines, calcium channel antagonists, anticoagulants, cyclosporine, and tacrolimus (100,101).

An important drug interaction of fluoroquinolones is the ability of ciprofloxacin to inhibit the metabolism of theophylline by CYP1A2 resulting in theophylline accumulation and toxicity (Table 30.2) (99). Seizures may occur at therapeutic levels of theophylline because of additive effects on the CNS (99). Exposure to quinolones may also increase the risk of Achilles tendon rupture and users of corticosteroids may be particularly vulnerable to this adverse event (102). According to a population-based study in the UK 2–6% of all Achilles tendon ruptures in people >60 years of age could be attributed to use of fluoroquinolones (102).

Biologics

Geriatric psoriasis is one of the key dermatologic conditions that physicians manage in their practice (103). In a U.S. population-based study the highest incidence of psoriasis was found in patients between 60 and 69 years of age (104). Traditional systemic therapies for psoriasis may not always provide improvement of the disease and the development of novel biological therapies provides new treatment options (105). Biologics for the treatment of psoriasis include T-cell modulating agents (e.g., alefacept), inhibitors of tumor necrosis factor-α (e.g., etanercept and infliximab), and inhibitors of interleukin-12 and interleukin-23 (e.g., ustekinumab) (105). Both alefacept and etanercept have been

TABLE 30.3
Prescribing in the Elderly: General Considerations

Take a careful drug history
Check for possible adverse drug reactions or drug interactions
Reduce the number of drugs administered simultaneously as much as possible
Start, when possible, with a small initial dose and titrate this dose to a clearly defined therapeutic response

studied in elderly patients with psoriasis (106,107). Alefacept was well tolerated and effective in elderly, obese, and diabetic patients with moderate to severe chronic plaque psoriasis (106). Similarly, no overall differences in safety were observed between older and younger patients with chronic, moderate to severe plaque psoriais treated with etanercept (107). So far no information is available regarding the risk of toxicity of the other biological agents in the treatment of elderly patients with psoriasis (60).

PRESCRIBING IN THE ELDERLY

Alterations in drug elimination and metabolism and higher prevalence of multidrug regimens put the elderly at increased risk of experiencing adverse drug reactions. In addition, drug response in the elderly is interindividually variable (12). There are no simple rules for prescribing that can apply to the elderly population in general (12). Still, some suggestions are made in Table 30.3 (12,18,19).

Estimates of nonadherence to medicines in the elderly with chronic conditions vary from 40% to 75% (108). Risk factors for nonadherence include inability to recall the medication regimen, medication costs, use of several physicians, polypharmacy and complicated drug regimens (109). Additional risk factors for poor medication management in the elderly include cognitive impairment and physical dependency (110). There are many unanswered questions regarding the most effective interventions to improve adherence to medicines (108). However, it is crucial to simplify the drug regimen as much as possible, that is, use of blister packs and prescription of drugs that can be taken at the same time of the day (19,108).

CONCLUSIONS

Some commonly prescribed dermatologic drugs, such as MTX and cetirizine may be eliminated more slowly in the elderly. Dosage reduction is recommended not only with these agents but any drug that is predominantly eliminated by the kidney. Potentially hepatotoxic drugs, such as MTX, itraconazole, and acitretin, should be used very cautiously in the elderly and liver function tests should be performed when these drugs are given to lower the risk of hepatotoxicity. However, further research is needed to determine how specific dermatologic drugs are handled by the elderly so that pharmacotherapy in this portion of the population can be improved.

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31 Sensitive skin: A valid syndrome of multiple origins*

Miranda A. Farage, Enzo Berardesca, and Howard I. Maibach

INTRODUCTION

The development of consumer health and beauty products routinely includes intensive premarket product testing intended to ensure that any marketed product is free of irritant potential. It is, nonetheless, not uncommon for postmarketing surveillance personnel to receive reports of unpleasant sensory reactions to such products not predicted by even the most robust development methodology (1). These sensory perceptions, often transient and unaccompanied by neither classical visible signs of irritation nor any immunologic response, have become a dermatologic phenomenon now most commonly known as sensitive skin.

Although initially believed to be an unusual reaction to common products, evidenced in only a small subset of consumers, epidemiologic surveys surprisingly find a high prevalence of self-perceived sensitive skin across the industrialized world (Table 31.1). In fact, most women in the United States, Europe, and Japan (representing the vast majority of patients queried to date) believe they have sensitive skin (2). In addition, the reported prevalences of self-perceived skin sensitivity have increased steadily over time, particularly among men (3).

Despite its apparently widespread existence, a functional understanding of this phenomenon has been elusive, primarily due to a multiplicity of (*i*) reported signs and symptoms, (*ii*) exposures that triggered unpleasant sensory effects, and (*iii*) testing methodologies that failed to produce any correlation with other methodologies or with consumer perceptions of skin sensitivity.

Self-reported sensitive skin comprised sensations described as prickly, burning, tingling, itching, stinging, or tightness (4). Objective signs are typically absent, but occasionally erythema (5), dryness, or rash is described, as are more intense inflammatory responses, such as wheal (6). The signs and symptoms that have been associated with sensitive skin have been reported to occur in conjunction with the menstrual cycle as well as subsequent to a cornucopia of possible triggers, such as weather conditions, air conditioning, cleaning products, personal care products, and clothing (5).

A variety of methodologic approaches (Table 31.2) have attempted to shed light on the underlying physiology of sensitive skin, with little initial success. The majority of research studies conducted have been small and not restricted to those verified to have sensitive skin (7). Most investigations have focused on objective assessment of physical effects rather than the sensory effects reported (8) and few reports quantified sensory effects or attempted to correlate sensory effects to the degree of irritation. In addition, few have attempted to evaluate possible confounders, such as endogenous hormones or concurrent irritant exposures.

Irritant testing also reveals profound interpersonal variability in individual response to specific irritants (9,10), even among chemicals with similar modes of action (11). Sizeable variation exists within the same individual at different anatomic sites (10), and even at the same anatomic site on symmetric limbs (12). In addition, many people who profess sensitive skin do not predictably experience visible signs of the sensations reported, whereas some who describe themselves as nonsensitive react strongly to tests of objective irritation (13).

Existing testing suggests that even when only those who identify themselves as having sensitive skin undergo testing, only a small subset of individuals will respond to any specific test. Response to one irritant has not shown to reliably predict sensitivity to any other and has not correlated well with evaluation of objective signs (14).

Ultimately, traditional irritant-testing methodologies have not proved to be good predictors of consumer response (1). With little meaningful basic science, the investigation of the phenomenon of sensitive skin has progressed to large extent on epidemiologic investigations based on self-perceived reports of sensitive skin. Self-reported data, however, has significant drawbacks. Patients may report underlying dermatologic conditions (e.g., rosacea or seborrheic dermatitis, which can also produce stinging sensations triggered by topical products) as sensitive skin (2,15). There are also psychologic disorders characterized by similar symptoms (e.g., cosmetic intolerance syndrome, dermatologic nondisease) (16). In addition, subject responses can be significantly impacted by the specific wording of survey questions.

Despite known physiologic characteristics of skin that could be expected to result in increased skin sensitivity, little consistency in self-perceived aspects of sensitive skin has been observed.

POTENTIAL CONTRIBUTORS TO SENSITIVE SKIN

A substantial diversity of exposures, both intrinsic and extrinsic, has the potential to contribute to perceptions of sensitive skin (Table 31.3).

Sensitive skin has historically been self-reported far more often in women than in men (Table 31.1). There is biological plausibility for greater sensitivity, as thickness of the epidermis was observed to be greater in males than in females ($P < 0.0001$), (17)

*This chapter is adapted from Ref. (73), with permission from Blackwell-Wiley.

TABLE 31.1
Percentage of Overall Skin Sensitivity Across the World

Country	Year[a]	Population	Overall Sensitivity**	Very Sensitive	Moderately Sensitive	Slightly Sensitive	Reference
Global Survey #1	2010	Male (47.5%) and Female (52.5%) 18–65 yr old N = 361	83.7	35.5	38	10.2	Farage, data yet unpublished
		Female only N = 191	83.1	14.0	36.6	32.6	
		Male only N = 172	81.5	6.6	37.1	37.7	
Asia		Male and Female 18–65 yr old N = 72	89.8	4.08	30.61	55.10	
Europe		Male and Female 18–65 yr old N = 94	87.67	16.44	42.47	28.77	
Latin America		Male and Female 18–65 yr old N = 78	85.42	18.75	41.67	25.00	
North America, Cincinnati, OH		Male and Female 18–65 yr old N = 122	76.84	7.37	35.79	33.68	
North America, other than Cincinnati		Male and Female 18–65 yr old N = 71	75.86	69	32.76	36.21	
Global, Survey #2	2010	Male (39.4%) and Female (60.6%) 18–65 yr old N = 218	85.1	9.3	46.5	29.3	Farage, data yet unpublished
USA, Mississippi	2010	Female 18–68 yr old N = 57	87.5	NA	NA	NA	Farage, data yet unpublished (with thanks to A. Wippel data collected from female staff at Hernando Junior and Senior High Schools, Hernando, Mississippi)
China	2009	Female 18–65 yr N = 408	23	2	5	16	(25)
Europe (total)	2007	Male and Female ≥ 15 yr N = 4506	74.7	13	25.1	36.6	(67)
Greece		Male and Female ≥ 15 yr N = 500	70	8.6	22.4*	29*	
Germany		Male and Female ≥ 15 yr N = 500	59	15.8*	20*	23.2	
Belgium		Male and Female ≥ 15 yr N = 500	60	10*	16*	4*	
Switzerland		Male and Female ≥ 15 yr N = 500	59	13*	18*	28*	

(continued)

TABLE 31.1
Percentage of Overall Skin Sensitivity Across the World (*continued*)

Country	Year[a]	Population	Overall Sensitivity**	Very Sensitive	Moderately Sensitive	Slightly Sensitive	Reference
Spain		Male and Female ≥ 15 yr N = 500	88	13*	20*	55*	
Italy		Male and Female ≥ 15 yr N = 500	90.6	17.4*	37.2*	36*	
Portugal		Male and Female ≥ 15 yr N = 500	86	16.2*	13.4*	56.4*	
France		Male and Female ≥ 15 yr N = 1006	82	12.1*	39.9*	30*	
France	2006	Female Adult N = 5074	61	NA	NA	NA	(66)
Japan	2006	Female Adults N = NA	~ 50	NA	NA	NA	(68)
USA (Ohio)	2006	Male 16%, Female 84% 18–65 yr N = 1039	68.4	4.9	23	40.5	(3)
USA (Ohio)	2006	Female N = 869 Mean age 35.1 yr Male N = 163 Mean age 38.1 yr	69 64.4	5.1 4.3	23.8 19	40.2 41.1	(63)
Greece	2005	Female Age NA N = 25	64	0	16	48	(35)
Greece	2005	Female with clinically diagnosed atopic dermatitis Age NA N = 25	100	44	36	20	(35)
France	2004–2005	Female 18–85 yr N = 400	85.4	30.5	NA	NA	(5)
Italy	2004	Male11.5%, Female 88.5% 18–80 yr N = 2101	59.9F	NA	NA	NA	(47)
France	March 2004	Male 41%, Female 59% ≥ 15 yr N = 1006	80.3	11.9	39.8	28.5	(69,70)
	March 2004	Female ≥ 15 yr N = 594	85.5	14.9	44.4	26.2	
France	July 2004	Male 48%, Female 52% ≥ 15 yr N = 1001	86.3	20.7	38.2	27.5	(70)
		Female ≥ 15 yr N = 521	91.2	28.2	41.1	21.9	

(continued)

Germany	2001	Male 39%, Female 61% Mean age 46 yr N = 420	75.0	17.6	29.5	28.1	(71)
		Female only N = 258	82.6	19.0	29.5	28.1	
UK	2001	Male and Female	49.6	8.1	NA	NA	(22)
		Female Age ≥18 yr N = 2046	51	10	NA	NA	
France	2000	Female Adult N = 310	90	~ 25	NA	NA	(72)
USA, California	1998	Female 18–54 yr N = 811	52	NA	NA	NA	(21)

*Percentages not reported but interpreted from bar graphs.
**Overall sensitivity percentages totals all respondents who reported any degree of sensitivity.
[a]Year of publication.

TABLE 31.2

Some Methodologies Used to Identify Sensitive Skin

Methodology	Sensory Affect Evaluated	Physical Effect Evaluated	Relevant Irritants	Advantages	Disadvantages
Lactic acid (2)	Stinging	None	Cosmetics, other personal preparations meant to be left on	Highly sensitive and specific[a]	Does not predict sensitivity to other irritants
Capsaicin (32)	Stinging	None	Cosmetics, other personal preparations meant to be left on	Sensitive, detection threshold well correlated (inversely) to perception of sensitive skin	Does not predict sensitivity to other irritants
Sodium lauryl sulfate (12)	Burning	Erythema	Industrial exposures, cleaning products	Cheap, quick, reliable assessment of individual susceptibility to specific irritant	Sensitivity to one irritant not predictive of general sensitivity, relationship to sensitive skin in question
Cross-polarized light (56)	None	Subclinical erythema	Any potential irritant	Permits detection of physical changes not apparent by standard visual scoring, noninvasive	Requires specialized equipment
Infrared thermographic scanner(73)	None	Temperature increases resulting from inflammatory processes related to skin injury	Any potential irritant	Noninvasive, objective, quantitative	Requires specialized equipment
Sebutape® (59)	None	Measurement of cytokines produced by injured skin	Any potential irritant	Noninvasive, objective, quantitative, potentially very sensitive	Requires training, specialized equipment. Utility for sensitive skin still unassisted

[a]Lactic acid test positive in 90% of women who claim sensitive skin. 2.

and hormonal differences that may produce inflammatory sensitivity in females have also been demonstrated (12,18). Irritant testing, however, for the most part finds no differences (10). In addition, self-reports of sensitive skin among men has steadily increased over the last decade (in concert with increased advertising of sensitive skin products marketed to men) (3), and a recent study in 1039 subjects found a 68.4% prevalence of self-reported sensitive skin, with no difference between men and women (3).

The physiologic changes of aging would also ostensibly predispose individuals to skin irritation (19); existing studies, however,

TABLE 31.3
Possible Contributors to Sensitive Skin

Factor	References
Female sex	(22)
Hormonal status	(74)
Environmental factors	(14)
Anatomic site	(3)
Age	(19)
Cultural expectations in technologically advanced countries	(62)
Fair skin, susceptible to sunburn	(53)
Susceptibility to blushing and/or flushing	(22)
Skin pigmentation	(75)
Thin stratum corneum	(7,20,76,77)
Decreased hydration of stratum corneum	(23,78,79)
Disruption of stratum corneum	(80)
Decreased barrier function	(41)
Increased epidermal innervations	(23,81)
Increased sweat glands	(20)
Increase neutral lipids and decreased sphingolipids	(82)
Decreased lipids	(28,83–86)
High baseline transepidermal water loss	(12)
Atopy	(35,60)
Incontinence	(87)

are ambiguous. Clinical assessment of the erythematous response to irritants in older people suggests a decrease in sensitivity with age (19), but a study of sensitive skin in 1039 subjects in Ohio found those over 50 years were more likely to claim sensitive skin than younger adults, particularly in the genital region (19).

Ethnicity and skin type are known to include pronounced differences in skin structure (7) and susceptibility to specific test irritants (12,20). Two large epidemiologic studies, however, reported no observed racial differences in reporting product sensitivity (21,22). Studies of racial differences with regard to irritants have yielded conflicting evidence (7,9,10).

While overall prevalence of skin sensitivity is similar across skin types and ethnic groups, some observable specific differences have been observed. Euro-Americans, relative to other ethnic groups, were found to have higher susceptibility to wind (21). Asians had higher sensitivity to spicy food, and Hispanics had relatively less reactivity to alcohol (21). African-Americans were more likely to report sensory response to stimuli, whereas Caucasians more often reported visual effects (14). African-Americans of both genders were more likely to report sensitivity in the genital area than other groups ($P = 0.0008$) (3).

Although topical health and beauty products and weather conditions are commonly associated with self-reports of sensitive skin (21,23), sensitive skin has also been reported to result from the environment (e.g., sun exposure, hot weather, cold weather, dry air, humidity, wind, air conditioning), health and beauty products (e.g., soap, shampoo, hair color, other hair products, eye cosmetics, facial cosmetics, facial moisturizers, facial astringents, facial cleansers, perfume, fragrances, body moisturizers, antiaging creams, sunscreen, deodorants, antiperspirants, talc), household items (e.g., cleaning products, dishwashing liquid, laundry detergent, fabric softeners), personal hygiene products (e.g., menstrual pads, pantiliners, incontinence pads, tampons, feminine wipes, douching products, toilet paper), garments (e.g., underwear, other clothing, rough fabrics), and personal health factors (e.g., the cyclic fluctuations of the menstrual cycle, stress). Using a fairly comprehensive questionnaire in China, a population with a comparatively low prevalence of self-perceived sensitive skin, every possible trigger suggested was claimed by at least a few respondents. The perceived prevalence of sensitive skin has been shown to be related to weather both temporally (significantly more women in France reported skin sensitivity in summer than in winter) (24) and geographically (women in China reported more sensitivity to hot weather, whereas women in the United States reported more sensitivity to cold) (25).

Most existing studies have been conducted on facial skin because of its sensitivity (stinging sensations, particularly, are readily elicited on facial skin (26) and because it is readily accessible for both visual (27) and biophysical assessments (28).

The face has demonstrated to be the most common site of skin sensitivity (Table 31.4), predictable physiologically due to the larger and multiple number of products used on the face (particularly in women), a thinner barrier in facial skin, and a greater density of nerve endings (16). The nasolabial fold was reported to be the most sensitive region (11) of the facial area, followed by the malar eminence, (11) chin, forehead, and upper lip (5,11). Saint-Martory found hand, scalp, feet, neck, torso, and back sensitivity followed by facial sensitivity in descending order of prevalence (5). Significant numbers of individuals experience sensitivity of the scalp (29,30).

In a study of 1039 men and women, 56.2% reported sensitivity of genital skin, (3) an area of particular interest since it is formed partially from embryonic endoderm, and therefore differs from skin at other body sites (13). A surprising 56.2% of responders claimed sensitive genital skin, with significantly more African-Americans than Caucasians (66.4%, $P < 0.0001$) claiming sensitivity of this area. Rough fabrics were found to be the most common offender for sensitive skin in the genital area (31).

Although both intrinsic and extrinsic factors undoubtedly influence individuals' decision to identify themselves as experiencing sensitive skin, no predictable constitutional factors have to date been identified (32).

The single consistency in the existing literature is that the majority of people in industrialized countries claim sensitive skin, and their perceptions related to the use of consumer product drives purchasing decisions; 78% of consumers, in fact, who profess sensitive skin report avoiding some products because of unpleasant sensory effects associated with their use (21).

These reports, obtained largely during postmarketing surveillance, were initially largely ignored due to a lack of dermatologic signs and the difficulty of quantifying subjective endpoints (33), with some investigators questioning sensitive skin as a genuine physiologic phenomenon. It was proposed, in fact, both in the popular media (34) and in the medical literature (2,22) that the increasing incidence of sensitivity represents a "princess and the pea" (34) effect, wherein it had become merely culturally fashionable to claim sensitive skin.

This "princess and the pea" (34) mindset of the medical community, however, hindered for some time a serious investigation of this ostensibly majority experience. In fact, much of the initial research was published in cosmetic trade journals (inaccessible through major databases) rather than leading dermatologic publications (4).

TABLE 31.4
Prevalence of Self-Declared Sensitive Skin at Specific Anatomic Locations

			Anatomic Location (%)			
Country	**Year**	**Population**	**Face**	**Body**	**Genitals**	**Reference**
USA	2009	Male (16%) Female (84%) Age NA N = 1039	77.3	60.7	56.3	(3)
USA	2009	Female N = 869 Mean age 35.1 yr Male N = 163 Mean age 38.1 yr	78.6 68.1	60.2 62	58.2 44.2	(63)
China	2009	Female 18–65 N = 536	21%	9%	6%	(25)
France	2008	Male and Female ≥ 15 yr N = 1011	NA	Scalp 47.4 Female 40.8 Male	NA	(29)
France	2004–2005	Female 18–85 N = 400	85	58 Hands 36 Scalp 34 Feet 27 Neck 23 Torso 21 Back	NA	(5)

Abbreviation: NA, data not available.

The phenomenon, however, has now been similarly recorded across four continents and in all industrial nations evaluated (21), with nearly identical prevalences in United States and Europe studies (68% (3) and 64% (35), respectively), lending credibility to consumer complaints. Furthermore, ongoing data collection increasingly supports the existence of an underlying physiologic cause.

THE ELUSIVE SOURCE OF SENSITIVE SKIN

Sensitive skin increasingly seems to include a set of seemingly disparate mechanisms.

Deficiencies in Barrier Function

Early studies suggested a link between sensitive skin and a disruption in barrier function (6,12,28,36,37), which has been shown to be a critical component of skin discomfort (6). Alterations in barrier function in sensitive-skin patients also have been observed (38,39).

Compromising the stratum corneum (SC) barrier in the subject with sensitive skin is believed to increase irritant permeability (40). The permeability barrier in the SC depends highly on lipid composition, a more accurate predictor of skin permeability than SC thickness or cell number (10). Derangement of intercellular lipids was associated with a decline in barrier function in sensitive skin (41); specifically, decreased neutral lipids and increased sphingolipids are associated with reduced barrier integrity (10). A weak barrier allows penetration of potential irritants (23), inadequately protects nerve endings, and facilitates access to antigen presenting cells, a mechanism which would support an association with atopic conditions (6). The SC barrier in sensitive skin has been demonstrated to be easily disrupted (6) with additional impairment of normal barrier recovery (42).

Measurement of barrier function using transepidermal water loss (TEWL; considered an indicator of the functional state of the SC (43)) have, however, largely failed to find significant and reproducible differences between sensitive skin subjects and controls (44). Pinto et al. (2011), however, using mathematical modeling of TEWL desorption curves, found significant differences between the skin of normal skin and that of sensitive skin in both evaporation half-life ($P = 0.005$) and dynamic water mass ($P = 0.0001$), suggesting that sensitive skin subjects do exhibit impaired barrier function (44). Moreover, daily use of moisturizer (four months in duration and expected to improve barrier function) did decrease skin sensitivity (2). Corneosurfametry, in addition, confirmed that subjects with a self-reported sensitivity to detergents had an increased reactivity to tested products as compared with the control group. It may be that a specific subgroup of sensitive skin subjects has SC compromise that causes a weakened resistance to surfactants (38).

Neurosensory Dysfunction

The variety of sensory manifestations that sensitive skin patients report, combined with the scarcity of objective signs, would seem strongly to indicate the presence of neurosensory defects in sensitive skin, presumed to be related to acceleration of nerve response, and therefore low-sensitivity threshold (32).

The pain sensations that are the hallmark of the disorder also imply possible integration dysfunctions in the central nervous system. Of the two studies reviewed that did evaluate the relationship between neurosensory responses and objective clinical irritation, and included only subjects with demonstrated sensory sensitivity, both showed a correlation between sensory and objective signs (38,39). In a study regarding sensitivity to facial tissue, which did not exclude nonsensitive individuals, sensory effects were demonstrated to be the most reliable measure of product differences (45).

Testing of sensory responses to specific irritants, however, have failed to provide consistent sensitivity patterns. For example, sensitivity to one irritant did not predict sensitivity to others (11). Green and Shaffer, in fact, found pronounced disparity in sensitive-skin subjects with regard to irritant response to just two chemicals (46). Although one study found that those who believed their skin to be sensitive were more likely to be stingers (59%) than nonstingers (48.9%) (47), there has been little consistent correlation observed between individual response to specific irritants in testing and self-perceived sensitive skin (32).

Recent research efforts, however, are homing in on the molecular basis for sensory hyper-reactivity. Transient receptor potential, vanilloid family 1 (TRPV1) is a nonreceptive, thermosensitive ion channel, which reacts to noxious stimuli, most notably noxious heat and low pH. TRPV1 is expressed on fibroblasts, mast cells, and endothelial cells; activation results in pain or pruritus with a burning component (48). TRPV1 is also dramatically upregulated by inflammatory mediators (48).

Interestingly, warmth detection thresholds were better preserved than other neural functions in patients with nerve damage, and epidermal innervation density correlated best to warmth detection thresholds (49).

Direct connections were observed between unmyelinated nerve fibers and mast cells; stress in animal models induces substance P (SP) in unmyelinated nerve fibers, which triggers mast cell degranulation with subsequent histamine release (50). Stress is commonly reported as a trigger for sensitive skin, and mast cell degranulation supported by finding that those with sensitive skin had a higher density of mast cells and size of lymphatic microvasculature (51).

Another recent study evaluated perception threshold measurement. Capsaicin (0.075%) and well-controlled electric currents were applied to the skin, and then sensory perception threshold was measured. Sensitive skin subjects had lower perception for c-fiber measurements than controls, suggesting the presence of a physiologically based neurologic instability with modulation of c-fiber nociception as a component (52).

Compound-specific Irritancy

The results of irritant testing suggest that some subset of sensitive skin is, in fact, related to individual sensitivity to specific irritants (53). Although studies have demonstrated that those with sensitive skin are capable of distinguishing products based on blinded sensory endpoints (8,11), a clinically satisfactory description of observed sensitivities remains out of reach.

Sensitive skin is typically absent of visual signs of irritation. It has been recognized, however, that skin sensitivity may represent subclinical trauma. Simion et al., by exaggerated arm-washing with synthetic detergent bars, observed signs that correlated statistically with sensory perceptions (dryness, tightness, and itching). In addition, consumers were able to reproducibly distinguish between test products purely on the basis of sensory effects (33).

Efforts have concentrated on optimizing ability to objectively assess physical signs of sensitive skin through three separate approaches as discussed below: exaggeration of test conditions, quantifying sensory responses, and increasing sensitivity of assessment of physical response.

Exaggeration of Test Conditions

A study of facial tissues, with and without evaluated four versions of facial tissues by employing repeated wiping in order to accentuate irritation. Affected skin had been compromised by tape stripping prior to the initiation of wiping. Erythema, as well as dryness, was evaluated daily. Statistical analysis revealed that the panelists' subjective product preferences were more consistent in distinguishing between the test product than were the visuals signs (erythema and dryness) (45).

A second method of exaggerating conditions in the testing of paper products is the Behind-the-Knee (BTK) protocol, which employs the popliteal fossa as a test site. BTK testing consists of the test product placed behind the knee and held securely by an elastic knee band, which in the course of daily activities adds a crucial mechanical friction component to the traditional testing (54).

Levels of irritation produced in BTK testing are consistently higher than those achieved with standard patch testing and have proved to be consistently reproducible (54). BTK testing, in conjunction with the other two approaches below, has proved useful in the development of potentially valuable protocols for sensitive-skin testing.

Quantifying Sensory Responses

One study tested feminine hygiene products according to four combinations of test conditions (wet/dry, intact/compromised skin) in parallel studies, which tested products by both traditional arm patch (55) and by BTK (56). Both studies evaluated observed erythema against a patient log of sensory effects. A significant correlation of reported sensory discomfort with mean irritant scores was observed. Skin sites where patients experienced burning, itching, or sticking had consistently higher mean irritant scores (55).

A companion paper, which evaluated products using BTK methodology but included sensory data collected from patient diaries in conjunction with the irritant testing, also observed correlation between sensory effects and mean irritant scores (55). Ultimately, eight separate comparison studies were able to statistically associate perceived sensory effects with an increase in irritant scores (54).

Increasing Sensitivity of Assessment of Physical Response

Our laboratory has also evaluated several new methodologies in an attempt to maximize the sensitivity of assessment of the physical response. Visual grading of erythema, the method of choice for many years, achieves a high degree of reproducibility with trained graders. A novel approach, however, utilized cross-polarized light, which allows visualization of the skin at a depth of 1 mm below the surface. Following minor irritation produced by low-level sodium lauryl sulfate (SLS), subsurface visualization provided no improvement over visual scoring. In BTK subjects, however, enhanced visual scoring through subsurface visualization detected significant differences in irritation produced by two

different test products, differences that were visible on the first day (56). Enhanced visual scoring was used successfully with both traditional patch testing, Forearm Controlled Application Test (FCAT), and BTK, providing a first link between sensory and physiologic effects. Subclinical changes were apparent after initial exposure; and enhanced visualization was able to correlate subclinical effects with consumer preferences between products (56), a correlation that had not been verifiable in prior testing (8).

Enhanced visualization was also evaluated in the genital area of symptomatic patients, demonstrating that cross-polarized light may be useful in diagnosing subclinical inflammation in ostensibly sensory vulvar conditions (57).

Changes in skin temperature related to inflammation have also been evaluated as a subclinical measure of skin irritation. Previous research has demonstrated a correlation between surface-temperature measurements and inflammatory response (58). A high precision, hand-held infrared thermographic scanner, recently developed, can conveniently measure changes in skin temperature in situ; observed skin temperature changes were both closely related to scores of erythema by visual scoring and also correlated to subjective sensory effects and product preferences (25).

An additional new technique in development uses an absorbent tape, which is applied to skin for 60 seconds and then removed (Sebutape® [CuDerm Corporation, Dallas, TX, USA]). Upon removal, several cytokines may be extracted from the tape and quantified. Compromised skin was shown to be significantly associated with increased levels of interleukin-1 alpha (IL-1α), IL-8, and the interleukin-1 receptor antagonist (IL-1RA)/IL-1α ratio. This technique may be helpful in the study of sensitive skin (59), particularly because numerous links between sensitive skin and atopy/allergy have been observed (1,19,22,35,60–62).

Cultural Influences

Lifestyle influences based on culture undoubtedly have an impact on the perception of sensitive skin. Cultural practices produce widely different exposures to potential irritants (32). For example, hygiene practices (use of douches, perfumes, medications, antifungal medications, and contraceptives) are the most common cause of vulvar irritation (18). Seasonal and geographic weather patterns vary. Older women were observed in one study to be more likely to report irritation due to incontinence products than younger women, who were more likely to report irritation due to tampons (19)—findings almost certainly based on culturally driven levels of exposure. Asians in one San Francisco study evidenced a greater skin reactivity to spicy food than Caucasians (21), another finding most likely related to a culturally higher exposure to spicy food.

Interestingly, the description of sensitive skin differs between ethnic groups. Caucasians claim visual (redness/swelling) effects *versus* African-Americans and Asians who claim more sensory (burning/stinging) effects (14).

The percentage of people who perceive themselves to have sensitive skin, however, has steadily increased in the United States and Europe with the increase in the media attention to this (Table 31.1). This is particularly evident among men (63), most likely related to an increase in the marketing of sensitive-skin products to men, driving a cultural acceptance of male sensitive skin. The fact that the majority of individuals who claim sensitive skin are women in the industrialized world also tends to support at least some psychosocial component.

Although the "princess and the pea theory," therefore, has effectively been put to bed by the ongoing unveiling of the physiologic mechanisms that underlie sensitive skin, the evidence suggests that the phenomenon of self-perceived sensitive skin has, at least in part, a cultural component driven by advertising and the perception that it is, in fact, socially acceptable to have sensitive skin.

APPROACHING SYNCRETISM

A few studies are beginning to examine possible interrelationships between disparate sensitive skin findings.

Following the observation that probiotic supplements can improve skin barrier function (64) and influence the pathogenesis of skin disease (65), Guéniche et al. evaluated a cream containing bifidobacterium longum lysate in the treatment of sensitive (reactive) skin, measuring skin sensitivity and susceptibility by a variety of methods (42). Ex vivo human skin explants were treated with both control and probiotic creams; treated explants were significantly improved with regard to measures of inflammation, such as edema, tumor necrosis factor alpha (TNF-alpha) release, decrease in TNF-alpha release, and mast cell degranulation as compared with controls. In addition, nerve cells, when cultured in vitro with the probiotic solution, released significantly less calcitonin gene-related peptide (CGRP) upon capsaicin exposure (42).

A subsequent randomized, double-blind clinical trial of a topical cream containing *Bifidobacterium longum* extract (n = 66) supported their preliminary in vitro findings. Although baseline sensitivity was similar between groups, volunteers who used the probiotic cream had significantly less skin sensitivity after 60 days of use than did the group who used the control cream ($P = 0.0024$) (42).

In addition, the number of tape strippings required to induce disruption of barrier function was significantly lower in the group that used the placebo cream as compared to the group who used the probiotic cream (42).

Because *B. longum* lysate was shown to both decrease capsaicin-induced CGRP and improve barrier function, the authors concluded that the decrease in skin sensitivity observed in the clinical trial was produced by a combination of both neuron reactivity and accessibility of neurons where barrier function is normalized (42).

Intriguingly, TRPV1 has recently been shown to play a role in barrier homeostasis as well. Topical application of TRPV1 activator capsaicin delays barrier recovery after tape stripping, whereas capsazepine, TRPV1's antagonist, stimulates recovery.

CONCLUSION

Sensitive skin, although now largely recognized as genuine syndrome of physiologic origin, is still largely a medical enigma with no correlation between sensory symptoms and subjective signs, universally accepted definition, and no reliable diagnostic test. Although it is clear that specific individuals clearly have heightened sensitivity to different kinds of sensory and physical irritants, observed reactions are not predictive of generalized sensitivity and the relationship between observed sensitivities is unclear (8,66).

As the underlying physiology of sensitive skin, through more sophisticated testing and evaluation of the phenomenon at a molecular level, begins to be revealed, it is becoming more and more probable that the phenomenon of sensitive skin is the product of multiple etiologies with multiple components. It may best be understood as several subsets of physiologic anomalies, all modulated by a wide variety of external exposures, existing in

multiple interdependent body systems, which interact to affect cutaneous responses (39).

Current needs are to continue to pursue reliably predictive diagnostic methodologies as well as those capable of detecting very subtle skin benefits or potential for adverse effects. In addition, epidemiologic data must continue to be bolstered by studies that build on what is known about the physiologic components of sensitive skin, such as to barrier function, neurosensory processing, and compound-specific sensitivity. More focused evaluation of the psychosocial aspects of sensitive skin is desirable as well.

The study of sensitive skin has emerged from the realm of fairy tales and has earned the respect due to a genuine physiologic disorder worthy of focused research. The fact that most western consumers are now "afflicted" begs a solution. The challenge of the future is to unravel the biological link between subjective clinical signs and their underlying physiology, thus to understand the etiology of this stubbornly enigmatic disorder.

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32 Dermatotoxicology of the vulva

Christina Y. Wang and Howard I. Maibach

INTRODUCTION

Vulvar toxicology is a unique subject because the vulva contains skin with specialized function and unique morphology. The skin of the vulva exhibits a higher degree of hydration, increased permeability, and is prone to irritation as manifested by edema, erythema, and/or corrosion (1,2). It is also the recipient of increased blood flow compared with skin on other sites of the human body (3), which can lead to altered absorption of, and reaction to, topical medications and other products applied to the area. Because of these characteristics particular to the vulva, its skin is subject to higher sensitivity to toxicities leading to dermatitis. On the other hand, despite its increased risk for dermatitis, the vulvar skin is pigmented, located in an occluded area, and structurally unique, thus, visually presenting dermatitic symptoms, such as erythema, in a widely varied fashion among different patients. This makes diagnosis via physical examination and visualization difficult and inconsistent, often presenting a conundrum for the physician trying to discover the etiology of the dermatitis and make the correct diagnosis.

PROPERTIES OF VULVAR SKIN

The vulva has unique skin properties that may predispose it to increased irritation and dermatitis (Tables 32.1 and 32.2). The vulva is subject to increased water loss and permeability to water, suggesting that vulvar skin is a less complete barrier and more prone to adversely react to irritants. The stratum corneum (SC) functions to retain water for the skin. The vulvar skin SC is thinner than other parts of the body, measuring 0.02 μm compared with 11.2 μm on the forearm, supporting the idea of its decreased barrier function. The vulva's increased water loss, and thus, permeability to water, is shown objectively by transepidermal water loss (TEWL) measurements by an evaporimeter. Mean TEWL in the vulva is higher at 1.42×10^3, compared with the lower measure of 8.68×10^2 in the forearm (4).

The amount of skin surface water loss is subject to more "bursts" (or varied increases) in the vulvar skin *versus* forearm skin. This varied water loss may be affected by occlusion and eccrine sweating on the vulvar skin, as in vulvar skin folds occluding on itself or garment occlusion. This variation can lead to data assessment complications in vulvar skin irritation studies (5). Researchers have tried to control for the occlusion factor on the vulva by drying out (via a desiccation chamber to absorb evaporated water) and comparing the capacitance (measure of skin hydration) on vulvar and forearm skin, measured by a capacitometer. Differences in TEWL and capacitance between forearm and dried vulvar skin were lessened but still apparent, suggesting that occlusion alone does not explain the vulvar skin's higher TEWL and that there are biological differences inherent in the vulva (6).

The vulva's higher capacitance, or skin hydration, leads it to have a higher friction coefficient, μ, which can be measured by the Newcastle Friction Meter. The vulva has a higher friction coefficient at 0.66 ± 0.03, compared with the forearm at 0.48 ± 0.01 (7). This higher friction coefficient leads to increased vulva skin friction irritation from mechanical trauma, such as occlusion, clothing, sexual activity, and moisture occlusion from incontinence, which increases skin moisture, resulting in an even higher friction coefficient (7,8). Of note, the higher incontinence-related friction coefficient comes even more onto the front stage in postmenopausal women who suffer from vulvar skin atrophy in addition to incontinence issues, predisposing them even more so to increased vulvar irritation and dermatitis (9).

The vulvar skin's higher hydration status (capacitance), higher TEWL, and decreased water barrier make it more permeable to polar irritants, such as maleic acid and benzalkonium chloride. The vulva has a greater than seven-fold increase in permeability compared with the forearm skin. The vulva showed a heightened irritation response compared with the forearm when exposed to the polar irritants maleic acid (20%) and benzalkonium chloride (17%) (1,10). Nonvulvar skin is less hydrated, less permeable to hydrophilic and polar compounds, whereas more permeable to lipophilic molecules (11). This is the basis for the development of nanosized drug delivery systems, such as dendritic core-multishell (CMS) nanotransporters (20–30 nm) and solid lipid nanoparticles (SLN, 150–170 nm) (12). The idea is that skin in much of the rest of the nonvulvar body has hydrophobic predominant characteristics, and absorption and delivery of hydrophilic drugs may be increased when placed in nanosized hydrophobic, lipophilic carriers, such as CMS and SLN (13–16). However, it is important to recall that the vulvar skin has somewhat opposite characteristics in that it has relatively increased hydration and permeability to hydrophilic compounds, and less to lipophilic ones. Thus, researchers need to bear in mind the unique vulvar skin in development of nanoparticle drug delivery systems for vulvar skin application, such as antifungals and podophyllotoxin, which can have useful applications in the genital area (16).

The vulvar skin has a higher blood flow and epidermal cell turnover rate compared with forearm skin (17). This may aid in its faster healing properties when comparing tape-stripped vulvar and forearm skin (18). Vulvar skin also shows higher extensibility without a comparable increase in elastic fiber network and retraction, likely needed in the physiologic changes necessary in childbirth (11,19). These unique properties most likely contribute to the vulva's ability to facilitate childbirth and postpartum healing, but may also predispose it to increased susceptibility to irritation.

TABLE 32.1
Unique Vulvar Skin Properties

1. Increased water loss transepidermal water loss
2. Increased skin hydration capacitance
3. Increased friction coefficient μ
4. Increased blood flow rate
5. Increased epidermal cell turnover rate
6. Increased skin extensibility

TABLE 32.2
Methods of Assessing Vulvar Skin Properties

1. Visual scoring system: skin irritation
2. Laser Doppler velocimetry: blood flow rate
3. Evaporimeter: transepidermal water loss – skin integrity against water loss
4. Capacitometer: skin hydration
5. Behind the knee test: frictional, mechanical irritation

ASSESSMENT OF VULVAR SKIN PROPERTIES AND IRRITATION

There are various methods of assessing the vulvar skin properties described above. Visual examination and scoring over vulvar irritation is one way, but it may be less sensitive and less able to capture all cases of vulvar skin irritation, especially low-grade dermatitis. The visual scoring system ranges from 0 to 4: normal skin, 0; slight redness, spotty or diffuse, 1; moderate, uniform redness, 2; intense redness, 3; and fiery erythema and edema, 4 (5,20). This method may not be as sensitive or consistent, as it is operator dependent.

More objective bioengineering instruments have been developed that aid in demonstrating and measuring the unique properties of vulvar skin. Laser Doppler velocimetry can show that blood flow is indeed increased in vulvar skin compared with forearm skin. Monochromatic light is subject to a light frequency change when reflected by moving blood cells, whereas stationary tissue does not show any frequency change. This instrument showed that the basal skin blood flow of vulvar skin was in fact significantly higher than in the forearm (17,20), confirming one vulvar skin characteristic that may aid in its increased healing capacity post-trauma, such as childbirth.

As described before, the vulvar skin has increased TEWL, which is a measure of SC integrity against water loss. TEWL is measured by an evaporimeter that consists of a hand-held probe that records the amount of water that evaporates from the skin surface, while maintaining the skin at a standard temperature. The vulva has increased skin hydration, or skin electrical capacitance, which is an indication of SC water content. It is measured by a capacitometer, which is a probe applied to the skin with slight pressure for three seconds, and the skin capacitance is reported as a digital readout (20).

The behind the knee test can assess for frictional effects and mechanical irritant properties of feminine hygiene products that contact the vulvar skin area. Recall that the vulva has an increased friction coefficient and susceptibility to mechanical trauma and skin irritation. Test materials are applied daily to the posterior knee area and held in place for six hours by an elastic knee band. Irritation is graded 30–60 minutes after product removal from behind the knee, using the four-point visual scoring system. Testing can use dry product on intact skin, dry product on compromised (tape-stripped) skin, wet product on intact skin, and wet product on compromised skin. Studies have shown that two applications of six hours each on intact skin are sufficient to ascertain product irritancy level. The test subject's reported sensory complaints, such as pain, stinging, and burning, may be associated with the degree of irritation seen on the objective four-point visual scoring (21,22).

DERMATITIS OF THE VULVA

Irritant Contact Dermatitis

In terms of overall contact dermatitis of the vulva, a German study in 1998 deemed 24–38% of noninfectious genital complaints were vulvar dermatitis. Other sources cite an incidence of 20–30% of vulvar contact dermatitis (3,23,24). Irritant contact dermatitis (ICD) is a nonimmunologic type of contact dermatitis. There are three types of clinical irritant reactions: acute, and chronic (cumulative) irritant dermatitis, and sensory irritation. Acute ICD results from exposure to a potent irritant, and can be thought of as analogous to a chemical burn. Chronic ICD results from cumulative exposures to weak irritants, and can sometimes be confused with immunologically based allergic contact dermatitis (ACD) (24), especially upon visual physical examination. Recent advances in molecular sciences allow for testing of mRNA from skin cells via tape-stripping to help distinguish between ICD and ACD, based on the presence of immunologic factors in ACD, and the lack thereof in ICD (25,26). Sensory irritation is characterized by a burning and stinging sensation due to an exposure, but is without detectable skin changes. The vulva can experience any of these three irritant reactions. Some chemicals, such as propylene glycol, can cause irritation (ICD) as well as sensitization (ACD) (23,27). Chronic ICD often involves both endogenous and exogenous etiologies. One endogenous factor is obesity, wherein increased skin folds increase moisture accumulation and the friction coefficient. Another endogenous cause involves the irritation of increased moisture and ammonia exposure with incontinence, which can be further worsened when coupled with vulvar skin atrophy in the postmenopausal patient population (9). Of note, there is evidence indicating that 46% of menopausal and perimenopausal women complain of vulvovaginal irritation symptoms (28). Some exogenous vulvar irritants include sanitary napkins (29), soaps, clothing, spermacides, and overly enthusiastic hygienic practices using soaps and antiseptic wipes (3,9,23). There are studies to develop anti-HIV vaginally applied microbicides, and there is hope that these will be an effective method of HIV transmission prevention, especially among women globally. It is important to keep in mind, however, with what is known about the unique vulvar skin permeabilities and sensitivities, the variable absorption and dermatologic tolerability of these potentially important topical drugs when used on the vulvovaginal skin area (30,31).

Allergic Contact Dermatitis

ACD is an immunologically mediated inflammatory skin reaction to an allergen in a sensitized person. As mentioned before, it is often difficult to differentiate between vulvar ACD and ICD, especially in light of the vulva's specialized, pigmented skin. In the acute ACD phase, vesiculation and severe pruritus can occur and

spread beyond the site of contact. The subacute or chronic phase produces more subtle symptoms, such as less severe pruritus and burning, redness, excoriation, scaling, and pigmentation changes with variable lichenification. ACD histology is similar to ICD, although acute cases may produce increased spongiosis (23).

Although there are not yet any widely used, definitive human predictive ACD tests, there are animal model assays for skin sensitization studies that involve guinea pigs and mice methods. The guinea pig model involves an induction phase where the test substance is exposed to the same skin area, then a rest period of at least seven days, followed by a challenge phase where a virgin skin site is exposed to the test substance and observed for reaction. In mice, the local lymph node assay (LLNA) is used. The LLNA involves an induction phase followed by injecting the mice with a label and then analyzing the draining lymph nodes for activation. Epidermal Langerhans cells are believed to take up antigen absorbed through the skin, travel to the skin area's draining lymph node, and then present the antigen to activate T cells, which then differentiate into allergen-responsive T lymphocytes (32,33).

Increased concentrations of allergenic antigens could potentially penetrate the vulvar skin since it has increased permeability and decreased barrier function (TEWL, capacitance), as discussed previously. This increased exposure to allergens may increase the risk of sensitization and ensuing ACD (11). Because of the vulvar skin's special properties and potentially increased risk of sensitization, ACD information from other body area skin, such as forearm, cannot be extrapolated to the vulva with utmost confidence. More conservative quantitative risk assessments may be needed when investigating vulvar ACD (33).

The modified human repeat insult patch test (HRIPT) helps take into account the vulvar skin's increased permeability to allergens. In a standard patch test, potential allergens are applied to normal skin on the back for two days under occlusion, with readings taken at days 2 and 4 (34). The original HRIPT had nine 24-hour applications of patches with 24-hour rest periods in between during the induction phase. The modified HRIPT increases the cumulative exposure by 67%, by increasing the number of applications to fifteen 24-hour patch applications (24 hours daily for five days, for three weeks, with the important rest periods in between to increase test effectiveness), thereby increasing the test sensitivity for evaluating specialized vulva skin. The five-day repeated steps mimic the use of some products, such as feminine hygiene products, which contact the vulva skin during the approximately five days of menses (35,36).

In a study of 135 vulvar skin symptomatic patients' patch-test results, 47% had at least one positive reaction, and 29% having a clinically relevant positive result (27). In another study of 50 women with vulvar skin pruritus, 52% had at least one positive patch test, with 16% having one or more relevant allergic positive reactions. Common allergens included cosmetics, medications, and preservatives (24). Fragrance mix positive patch testing occurred in 11%, with clinical improvement of vulvar dermatitis when perfumed products were avoided, such as scented feminine hygiene products. Another 11% of positive patch tests were to product preservatives formaldehyde and its releasers, such as Quaternium 15 and DMDM Hydantoin, found in creams and hygiene products applied to the vulvar and other areas (37).

There is an abundance of common vulvar allergens available to patients over the counter. These include topical anesthetics used in vaginal preparations, such as benzocaine, topical antibiotics, such as neomycin, topical antifungals, such as nystatin, and topical steroids, not to mention the preservatives often used in these products, as discussed above (2,38). The ever rising popularity of herbal remedies opens the door for a new host of potential vulvar skin allergens. Chamomile sensitized 2.9% of patients, arnica 2.1%, and propolis 2.5% of patients. The extent of sensitization potential may depend on herbal dose, purity, and quality (3). Oral ingestion of herbal products may also affect the vulva upon excretion of said products via urination. A patient was found to have patch tested vulvar skin ACD from drinking huge quantities of peppermint oil-containing herbal tea, daily for six years straight. Contact with oral tissue may have been too short in duration, or the metabolization of the substance prior to urinary excretion could have caused symptoms in the vulvar but not oral skin areas. Nonetheless, once the patient stopped drinking peppermint tea and avoided all other peppermint-containing products, her symptoms improved (39).

Many other consumer products that contact the vulva may contain potential allergens. Dark clothing, such as underwear, can harbor paraphenylenediamine (PPD)-containing dye and formaldehyde, known sensitizers. It is interesting to note that there have been many reports of ACD to the PPD contained in dark henna dyes used for temporary skin tattoos (40–42). Although these henna tattoos are usually placed on nongenital areas, one should keep in mind that there are honored traditional tattoos placed in all different body areas, including genital sites, respecting the ethnicity and heritage of some cultures, such as in the South Pacific Islands (43). In modern and permanent tattoos, various ink pigments and ingredients have been known to cause ACD and photoallergic dermatitis, to name a few skin reactions (40,44–46). Patients may choose various sites for pigment introduction, in some cases, including the genital areas. In cases of the vulvar skin area, one must bear in mind its unique properties, which lead to potential increased sensitization and ACD, in reaction to the myriad of consumer products and procedures used in, on, and around the vulvar area.

Photoirritation and Photoallergic Dermatitis

Photoirritation, or phototoxicity, is a nonimmunologic skin irritation requiring an inciting chemical plus light. The skin reaction resembles a sunburn (47). Photoallergic dermatitis is a subtype of photosensitive dermatitis, resulting from ultraviolet (UV)-induced excitation or activation of a chemical applied to the skin, after a period of sensitization. These reactions are delayed, manifesting days to years after the UV exposure (48). Given the relatively sun-protected location of the vulva, there is little data available describing these reactions.

CONTACT URTICARIA

Nonimmunologic Contact Urticaria

The contact urticaria syndrome is an immediate contact reaction consisting of inflammatory reactions that appear, usually within minutes, after contact with an eliciting substance. The reaction includes wheal and flare with transient erythema, which may lead to eczema. The most common subtype is the nonimmunologic contact urticaria (NICU), which occurs without prior sensitization. This reaction remains localized, does not spread to become generalized urticaria, nor does it cause systemic symptoms. The reaction varies from erythema to an urticarial response, depending

on dose, surface area exposed, mode of exposure, and the particular substance (49,50). One can test for a substance's potential for causing such immediate reactions by applying the substance to a guinea pig ear lobe, and if it becomes edematous and erythematous, then the substance is capable of causing a contact urticarial reaction. Edema can be quantified by measuring ear lobe thickness changes with a micrometer caliper. Guinea pig ear lobe biopsies characteristic of NICU demonstrate dermal edema and intra- and perivascular infiltrates of heterophilic (neutrophilic in humans) granulocytes (50,51).

In humans, the open test can be used to assess for NICU; 0.1 mL of test substance is applied to a 3 × 3 cm area of skin on the upper back or extensor surface of the upper arm.

The area is observed for 60 minutes, looking for edema, erythema, or small intraepidermal spongiotic vesicles typical of acute eczema, denoting a positive result. If the test is initially negative on nondiseased skin, another testing is done on affected skin (50,52). Unfortunately, thus far, there is scant data regarding vulvar skin reactions of this nature.

Immunologic Contact Urticaria

Immunologic contact urticaria is an IgE-mediated reaction consisting of a local wheal and flare, which in some cases, escalates into asthma, allergic rhinitis, and/or conjunctivitis, anaphylaxis, and rarely, death. Diagnosis can be made by using the open test method of skin testing, using extremely diluted solutions under strict protocols and precautions (53). Again, little is documented regarding vulvar skin reactions of this type.

CONCLUSION

The vulva contains skin that has unique morphology and properties, including increased permeability, hydration, friction coefficient, and susceptibility to irritation from some chemicals and physical trauma. However, it also has an increased blood flow, cell turnover rate, and extensibility, making it an ideal skin to allow for childbirth and healing thereafter. The vulva has a unique response to irritants and allergens. Some substances are more permeable on the vulva (relatively more polar and hydrophilic), whereas they are less so on other skin, such as the forearm. Some chemicals induce higher irritation on the vulva, whereas others induce less irritation compared with that on the forearm skin. Future studies, and drug and transdermal drug carrier development, should consider the special characteristics of the vulva and use appropriate testing methods and targeted biochemical properties in assessing and accessing this unique area of skin.

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33 Human scalp irritation related to arm and back*

Hongbo Zhai, Rolf Fautz, Anne Fuchs, and Howard I. Maibach

Large-scale irritation tests for dermatologic and cosmetic products have been conducted on the back and arm. Like the face, the scalp is frequently a target of topical products, such as drugs and cosmetics. Yet, shampoos often contain potential irritants. The scalp may hide classical irritation morphology (erythema and edema). However, a few investigations documented the scalp's structure and functions (1–3). In particular, data comparing reactions to surfactants between scalp and back and arm are limited.

Typical exposure to surfactants by the consumer is repetitive, for brief periods, and via open application, such as hand washing or personal cleansing. Therefore, commonly used patch tests that use surfactants under occlusive condition to determine irritation potential may not express their irritation potential under typical consumer use conditions. An open-test method that mimics real-life application of surfactants is required.

This study used our previously established open-application model (4) with a modification to testing potential irritancy of a "gold standard" model irritant, sodium lauryl sulfate (SLS) on human scalp (5). The sensitivity of responses between scalp and back and arm were also explored.

MATERIALS AND METHODS

Subjects

Ten healthy, Caucasian, adult bald men (mean age 56±9 years) were enrolled. Subjects were healthy with no obvious skin disease or known history of atopic dermatitis. The University of California at San Francisco Human Research Committee approved this study.

Surfactant

SLS (99% purity, Sigma Chemical Co., St. Louis, MO, USA) was freshly prepared in deionized water at 20% (w/w) concentration.

Experimental Design

This study was randomized (each test area was chosen in a random manner). Each volunteer served as his own control between the different test regions.

General Procedure

Three test areas were designated separately on volar forearm, upper back, and scalp. The designated test area was approximately 3 cm^2. Basal values of instrumental measurements were taken on each test site prior to SLS treatment. A technician conducted five successive washing: for each wash, the technician pipetted 1 mL of 20% SLS solution into a glass cylinder (2 cm of diameter) placed on the designated area with hand pressure that prevented the cylinder leaking. The test area was then rubbed with a Teflon Policeman scrubber (Fisher Scientific, Pittsburgh, PA, USA) for 1 min. At the end of scrubbing, the solution was absorbed dry with a plastic pipette and then the test area was blotted by gently applying paper tissues. After a 5-min rest, the procedure was repeated for four more times for a total of five times. Skin-irritancy assessments by visual scoring and instrumental measurements were made at 30 min and 24 h thereafter.

At the end of all measurements, one stripping of stratum corneum (SC) was taken by using tape disks (D-squame1, Cuderm Corporation, Dallas, TX, USA) from each test area. Additionally, three untreated (normal) skin areas were stripped with D-squame1 disk as blank controls from the volar forearm, upper back, and scalp of each subject. These disks were for the squamometric analysis. Details of squamometric analysis have been described previously (4,6–8).

Visual Scoring

Visual scoring (VS) was judged according to the following rating scale (4–8): Erythema: 0 = no redness; 1 = very slight redness; 2 = slight redness; 3 = moderate redness; 4 = strong redness; 5 = very strong redness; 6 = extreme redness.

Instrumental Measurements

Transepidermal water loss (TEWL) was assessed by a Tewameter (TM 210, Courage & Khazaka, Cologne, Germany, and Acaderm Inc., Menlo Park, CA, USA). TEWL documents the integrity of SC water-barrier function and is a sensitive measure of surfactant-induced skin barrier alteration (9). The measuring principle and standard guidelines are published (10). TEWL values were expressed as $g/m^2/h$.

*This chapter has been adapted and updated from Zhai H, Fautz R, Fuchs A, et al. Human scalp irritation compared to that of the arm and back. Contact Dermatitis 2004; 51: 196–200, with permission from Blackwell-Wiley.

Skin capacitance was measured by a Corneometer (CM 820, Courage & Khazaka and Academ Inc.). Capacitance is a parameter of SC hydration (or water content). The measuring principle and methods are described elsewhere (11). It was expressed digitally in arbitrary units (a.u.).

Erythema was quantified by skin color reflectance measurements using a colorimeter (Chroma Meter CR 300, Minolta, Osaka, Japan, and Academ Inc.). The a* value represents the color spectrum from total green to pure red and correlates closely with erythema (9,12). Standard guidelines and measuring principles have been published in detail (13).

The measurements were conducted in a room with daily ranges of relative humidity (RH) from 50.8±3.8% and temperature from 20.1±1.3°C. These values (RH and C) were recorded during the experimental period. Each subject rested at least 30 minutes for acclimation before measurements.

Statistical Analysis

Statistical analysis was performed using a computer program Sigmastat (SPSS Science, Chicago, IL, USA). One-way repeated-measures analysis of variance evaluated the differences for values of TEWL, capacitance, and a*. *t*-Test was performed with values of C*. The Friedman-repeated measures were performed for the nonparametric visual scores (erythema). Wilcoxon-signed rank test was performed for the nonparametric data (intercorneocyte cohesion and the amount and distribution of dye found in cells). All tests of comparisons were two sided at a significance levelof 0.05.

RESULTS

Visual Scores

VS was significantly increased ($P < 0.05$) on the back in comparison to the forearm at both time points of 30 minutes and 24 hours post-SLS treatment. The VS on the scalp was higher than on the forearm but did not reach statistical significance.

Instrumental Evaluations

a Values

a* was significantly increased ($P < 0.05$) on the back in comparison to the forearm as well as to the scalp at the time points of 30 minutes and 24 hours post-SLS treatment (Fig. 33.1). a* showed a slight increase on the scalp when compared with the forearm but did not reach statistical significance.

Transepidermal Water Loss

TEWL was significantly increased ($P < 0.05$) on the back in comparison to the forearm at 30 minutes post-SLS treatment (Fig. 33.2). After 24 hours, there were no statistically significant differences between the sites.

Capacitance

Capacitance was significantly changed ($P < 0.05$) on the back in comparison to the forearm at 30 minutes post-SLS treatment. Also, capacitance was significantly changed ($P < 0.05$) on the scalp in comparison to the forearm at 30 minutes post-SLS treatment. After 24 hours, there were no statistical significant differences between the sites.

*Chroma C**

The Chroma C* was significantly increased ($P < 0.05$) on the SLS-treated sites in comparison to control sites (normal skin) except on the back.

Squamometry

Dye Fixation

The dye fixation at all SLS-treated sites was significantly increased ($P < 0.05$) in the cells in comparison to control sites (normal skin).

Cohesion Assessment

All SLS-treated sites showed a significantly greater loss of cell cohesion ($P < 0.05$) in comparison to control sites (normal skin).

Change Ratio % and Sensitivity to SLS Treatment

The "change ratio %" was calculated with the following formula: Change ratio % = (treatment – baseline)/baseline × 100% or = (time point post-treatment – baseline)/baseline × 100%.

Table 33.1 summarizes the change ratio % of all parameters. Rank of changes (sensitivity) to SLS treatment for a* from different regions: at 30 minutes post-treatment was back > forearm > scalp; after 24 hours post-treatment was back > scalp > forearm. Rank of changes (sensitivity) for TEWL: at 30 minutes post-treatment was back > scalp > forearm; after 24 hours post-treatment was back > scalp > forearm. Rank of changes (sensitivity) for capacitance: at 30 minutes post-treatment was scalp > forearm > back; after 24 hours post-treatment was back > forearm > scalp. Rank of changes (sensitivity) for Chroma C* was forearm > scalp > back. Rank of changes (sensitivity) for dye was forearm > scalp > back. Rank of changes (sensitivity) for cohesion was back > scalp > forearm.

DISCUSSION

Male-pattern baldness (androgenetic alopecia) is a common hair loss disorder. Kligman (14) noted histopathologic inflammatory changes in the upper dermis in male-pattern baldness, but most of the skin surface is clinically devoid of inflammatory change. Furthermore, O'goshi et al. (2) confirmed that SC function of the skin in male-pattern baldness was intact without any impairment in terms of barrier function or water-holding capacity. Hence, we chose these subjects as a suitable model to determine the response to SLS on their scalp.

Here, most parameters (VS, a*, TEWL and cohesion) showed that the back was most sensitive to SLS challenge. Thus, these results support the current standard skin-compatibility testing procedure, employing the back for potential irritation testing of hair-care products. However, capacitance data were complicated to interpret because this mainly reflects skin hydration rather than impairment of the water barrier. A previous study indicated that removing skin surface lipids from the scalp and forearm by acetone markedly reduced skin-surface hydration values (2).

Squamometry, a sensitive assessment for detecting surfactant-induced subclinical irritation (4,6–8), demonstrates superiority in the evaluation of efficacy of moisturizers and barrier creams

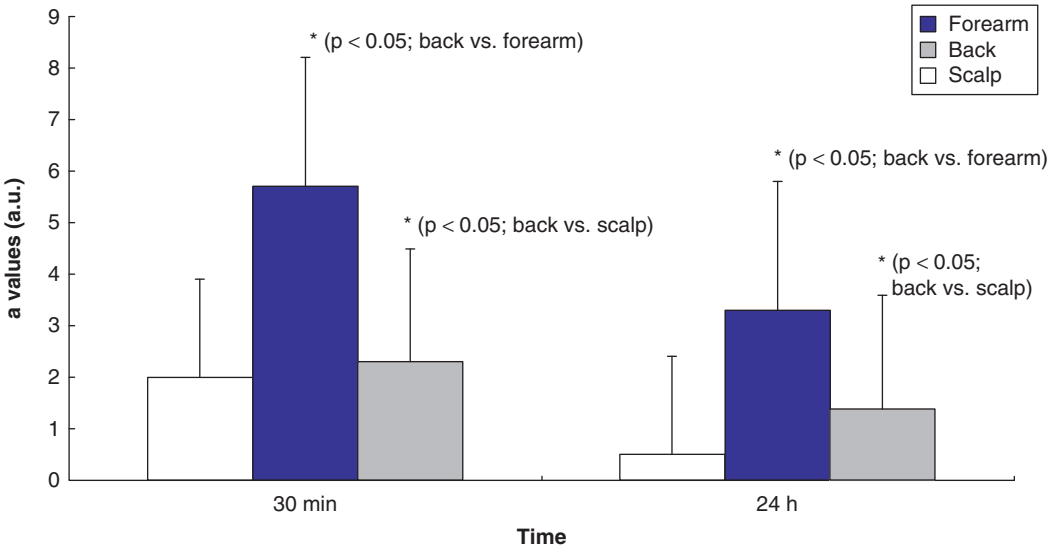

FIGURE 33.1 Comparison of a* at different regions at 30 minutes and 24 hours after sodium lauryl sulfate treatment (data were subtracted from baseline).

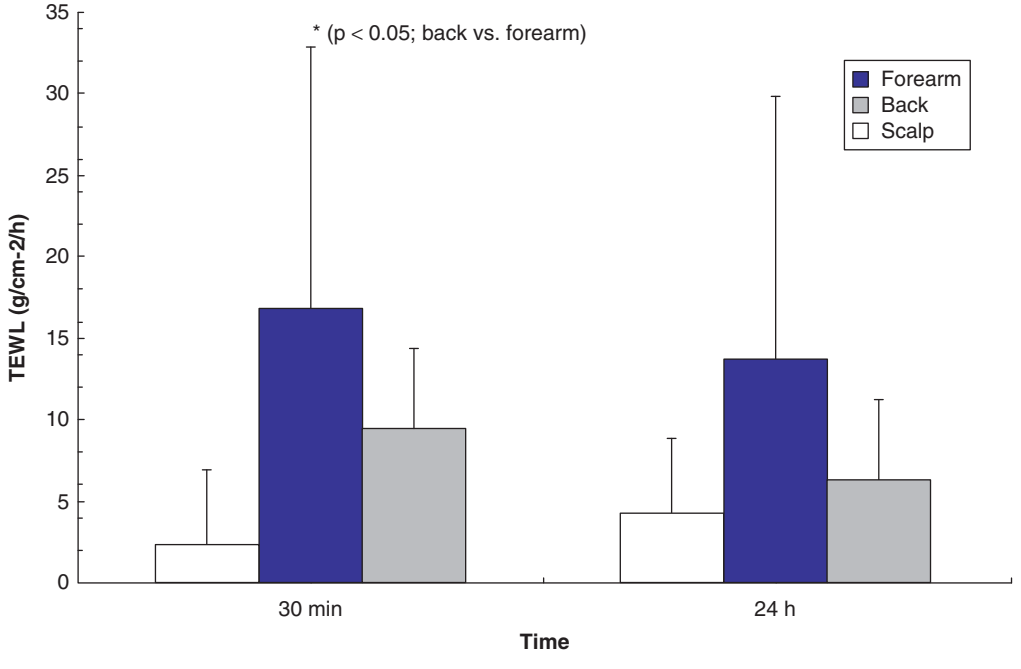

FIGURE 33.2 Comparison of transepidermal water loss at different regions at 30 minutes and 24 hours after sodium lauryl sulfate treatment (data were subtracted from baseline).

TABLE 33.1
Change Ratio Percentages of All Parameters

Parameters	Time	Forearm (%)	Back (%)	Scalp (%)
a Values	30 min	20.0	66.3	18.5
	24 hr	5.0	38.4	11.3
TEWL	30 min	30.6	240.0	99.0
	24 hr	58.3	195.7	66.7
Capacitance	30 min	−11.0	5.9	14.1
	24 hr	3.0	4.9	−1.0
Chroma C*		67.7	20.0	38.5
Dye		76.9	31.6	68.8
Cohesion		140.0	233.3	190.0

against a surfactant irritant (15,16), as well as in screening hydrating products (17). Data of dye and Chroma C* were consistent but different from cohesion, because there are two mechanisms between cohesion and dye fixation: cohesion with protein and lipid metabolism; and dye fixation with protein only (4). For example, if the surfactant has sufficient solvating properties, it might influence cohesion. Another point is the way that surfactants are applied. When applying the tested surfactant by washing, the surfactant might cut the cells (protease mechanism) in the first layers (because of its irritancy) and the friction of the wash-remove cells; then they are removed with the rinse procedure.

Recent data indicated that there was no difference in the biophysical parameters obtained for the barrier function or water-holding capacity of the SC between lesional skin of alopecia

areata and freshly shaved scalp skin (2). In general, investigations on the functional aspects between bald scalp and shaved healthy scalp skin are limited. We have minimal data on thresholds for irritation at varying anatomic sites. Percutaneous penetration (18) and irritation (19) differs from site to site. We would assume that irritation thresholds also differ but have an inadequate database to so confirm. Data having been obtained for acute irritation with a hydrophilic surfactant cannot be, at this time, equated with either hydrophobics or other mechanisms (sensory irritation, cumulative irritation, or allergic contact dermatitis). Because this was testing on the scalp of male-pattern baldness subjects, we did not wish to overinterpret the data to the hairy scalp. This information provides the potential for planning the next level of use-type study. Other researchers (20,21) provided detailed data on regional variation in reactivity of the human face, neck, and forearm.

Recently, Fujita et al. (22) compared the reactions of sensory irritation caused by hair-dye on different regions of the body and indicates that the cervical region is an appropriate site for testing the sensory irritation of a hair-dye. We recent published additional human experimental data, comparing the reactions of nonimmunologic contact urticaria on scalp, face, and back (23). Taken together, we encourage investigators into this relatively neglected but clinically important arena.

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34 Functional map and age-related differences in human faces: Nonimmunologic contact urticaria induced by hexyl nicotinate

Anaheed Heydari, Panthea Heydari, Slaheddine Marrakchi, and Howard I. Maibach

INTRODUCTION

Age-related and regional variation studies of the human skin reactivity to various irritants have been reported (1–5). A marked variation in the various areas of the face in reactivity to benzoic acid has been documented by Shriner and Maibach (6).

In the present study, hexyl nicotinate (HN), a more lipophilic compound than benzoic acid, was used to induce nonimmunologic contact urticaria (NICU) in the same sites documented by Shriner and Maibach (6). Blood flow changes were recorded to determine the potential regional and age-related differences in cutaneous vascular reactivity to HN.

MATERIALS AND METHODS

Subjects

Two age groups were studied: 10 healthy volunteers in the young age group, 29.8 ± 3.9 years, ranging from 24 to 34 years, and 10 in the older group, 73.6 ± 17.4 years of age, ranging from 66 to 83 years.

Exclusion criteria were a history of atopy and current antihistaminic drug use.

All the volunteers gave written consent and the study was approved by the local ethical committee.

Methods

Eight regions—forehead, nose, cheek, nasolabial and perioral areas, chin, neck, and volar forearm—were studied in terms of stratum corneum (SC) turnover (dansyl chloride test) and pharmacodynamic response to HN.

Dansyl chloride 5% in petrolatum was applied for 16 h on the eight locations of the skin using 8 mm Finn Chamber aluminum disks (Epitest Lid, Oy, Finland) (7).

After dansyl chloride patch removal, the subjects were allowed to acclimate to the examination room for 15 min, then baseline measurements were taken on the contralateral locations.

Baseline measurements of the cutaneous blood flow (LDF) were taken using a laser Doppler flowmeter (laser Doppler flowmetry blood flow monitor, MBF3D, Moor Instruments, England) (8). Blood flow measurements were not taken on the upper eyelid because of the potential effect of the laser beam on the retina. Blood flow was monitored at one measurement per second for 30 seconds and the values were averaged.

Using a saturated absorbent filter paper disk (0.8 cm diameter) (Finn Chamber), HN 5 mM in ethanol was applied on the eight skin areas for 15 seconds to elicit NICU. Then blood flow measurements were taken every 10 minutes for 1 hour to detect the maximum vascular response of the skin to HN.

SC turnover was determined by detecting fluorescence on each skin site everyday using a ultraviolet (UV) lamp. The period for the fluorescence to disappear was considered as the SC turnover. Tagami (9) showed that in addition to the high metabolic activity corresponding rapid turnover of the SC, the face comprises a unique portion of the body where the openings of the distinct anatomic structures, such as the eye, nose, ear, and mouth are densely populated.

Room temperature and relative humidity were recorded each time a subject was studied. Room temperature during the young group study (20.3°C ± 2.3°C) was significantly ($P = 0.042$) lower than during the older group study (22.1°C ± 2.3°C).

Relative humidity during the young group study (52.6 ± 3.8) was significantly higher ($P = 0.009$) than during the older group study (46.5 ± 5.5).

Statistical Analysis

To compare the measurement of the various skin sites within each group, the analysis of variance test for analysis of variance was used. The two-tailed Student's *t*-test for unpaired data was used to compare the differences between the two age groups.

RESULTS

Baseline to Peak Changes

Cutaneous reactivity to HN was assessed by the baseline to peak changes (peak = maximum LDF – baseline LDF). In some investigations, area under the curve was also considered to assess these changes (6,10,11), but as it was correlated to peak values (6), only the baseline to peak changes (peak) were considered in our study.

Comparison Between Regions

In the young group, the perioral area, followed by the neck, was the most sensitive to HN. The perioral and the nasolabial areas, the

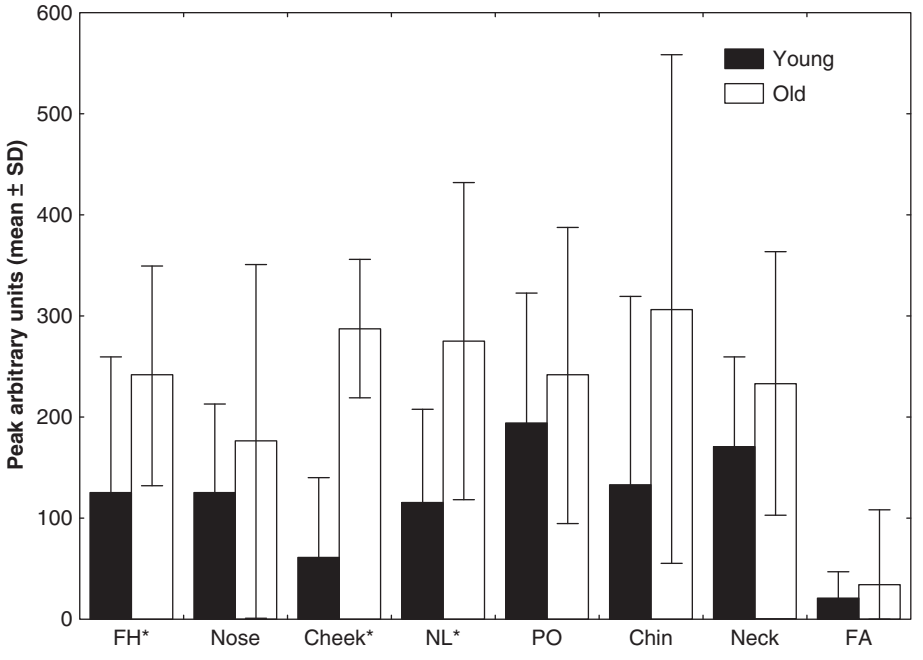

FIGURE 34.1 Baseline LDF to peak changes. Regional variation in the young and old groups and age-related differences. *The regions where the difference between the two age groups was significant ($P < 0.05$). *Abbreviations*: FA, forearm; FH, forehead; PO, perioral area; NL, nasolabial area; SD, standard deviation.

nose, forehead, and the neck were more sensitive than the forearm ($P < 0.05$). Perioral area ($P = 0.012$) and the neck ($P = 0.009$) were more sensitive than the cheek (Fig. 34.1).

In the older group, all the areas of the face were more sensitive than the forearm. The chin followed by the cheek and the nasolabial area was the most sensitive. However, no difference in reactivity to HN was found between the various areas of the face.

The forearm was the less sensitive area in both the groups.

Comparison Between the Two Age Groups

Peak values were higher in the older group in three areas: forehead ($P = 0.047$), cheek ($P < 0.001$), and nasolabial area ($P = 0.012$) (Fig. 34.1).

Stratum Corneum Turnover

Comparison Between Regions

Facial skin in adults consists of much thinner SC showing rapid turnover time than other anatomic locations. Although the facial skin shows rather poor barrier function, it is covered by well-hydrated soft and smooth surface (9). Compared with other body locations, except that of the neck, the face is covered by morphologically and functionally distinct SC (9). Studies (12) also indicate that barrier function of the SC was best on the cheek. A positive correlation was shown (Fig. 34.2) between the age and the size of corneocytes on the cheek (b), nasolabial fold (c), and chin (d) (12).

The SC turnover was slower in the nasolabial area and the forearm in both age groups.

The fastest SC turnover was shown in the perioral area and the chin in the young and in the chin and the forehead in the older group.

In the young group, nasolabial area and forearm SC turnover was significantly slower ($P < 0.05$) than forehead, cheek, perioral area, and the chin. The SC turnover was slower in the nose when compared with forehead ($P = 0.028$), perioral area ($P = 0.016$), and the chin ($P = 0.015$). The SC turnover was slower in the neck than the perioral area ($P = 0.004$) and the chin ($P = 0.029$).

In the older group, the forearm and the nasolabial area demonstrated a significantly slower SC turnover than the forehead ($P < 0.001$ and $P = 0.008$, respectively), the nose ($P < 0.001$ and $P = 0.025$), the cheek ($P < 0.001$ and $P = 0.011$), the perioral area ($P < 0.001$ and $P = 0.023$), the chin ($P < 0.001$ and $P = 0.001$), and the neck ($P < 0.001$ and $P = 0.013$).

Comparison Between the Two Age Groups

In the nose and the neck, the SC turnover was significantly ($P < 0.05$) slower in the young group than in the older group (Fig. 34.3).

DISCUSSION

Vascular Response to HN: Peak, Blood Flow, and Transepidermal Water Loss

In the young group, the highest vascular responses to HN were perioral area and the neck. In the older group, the chin, cheek, and nasolabial area showed the highest skin reactivity to HN (Fig. 34.4).

This difference between the two age groups might be partly explained by the enlargement of the sebaceous glands in the elderly (13). The ultraviolet A has been reported to induce sebaceous gland hyperplasia (14), which might lead to the enlargement of the sebaceous glands in the face when compared with other areas (15,16) and in the elderly when compared with the younger subjects (13,17).

Blood flow level was highest in the lips, followed by the cushion of the third finger, nasal tip, earlobe, palm, cheek, back of the third finger, and forehead (18). Appendages may be an important factor in the HN absorption, as the areas in the older group, where peak

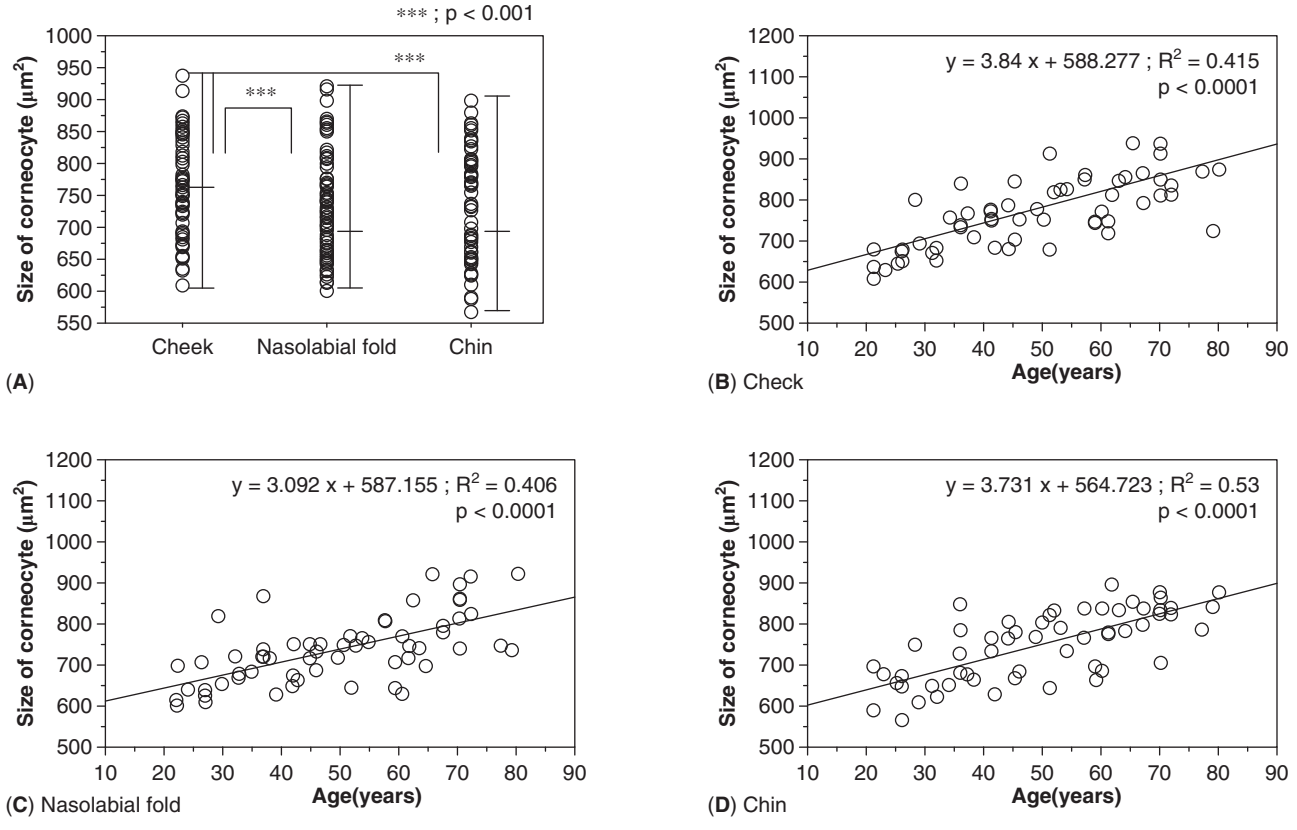

FIGURE 34.2 The size of superficial corneocytes on the forehead, nose, cheek, nasolabial fold, and chin. The size of superficial corneocytes on the nasolabial fold and chin was significantly smaller than that on the cheek (**A**). There was a definitely positive correlation between the age and the size of the corneocytes on the cheek (**B**), nasolabial fold (**C**), and chin (**D**).

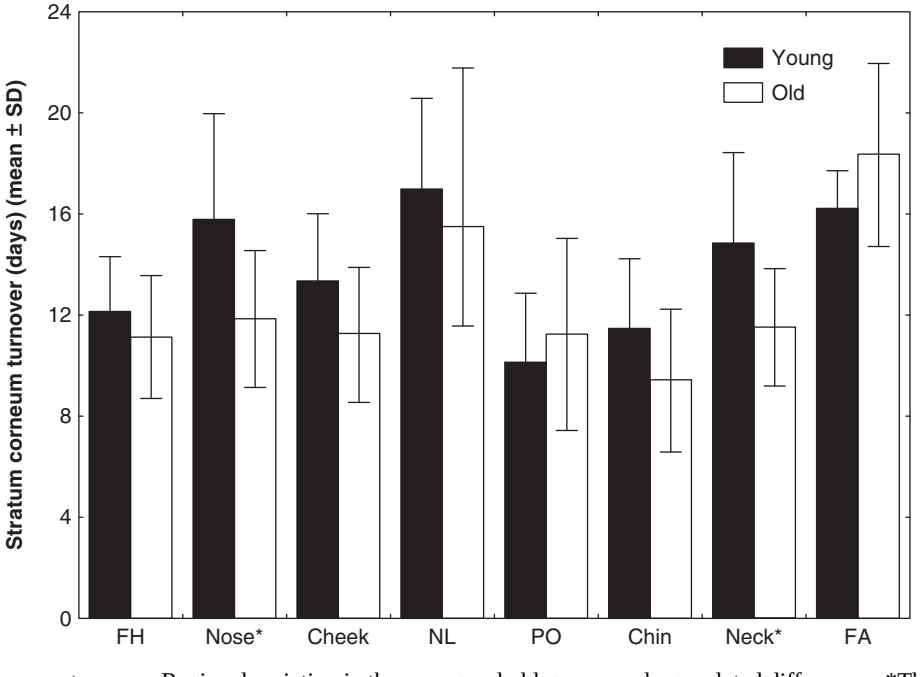

FIGURE 34.3 Stratum corneum turnover. Regional variation in the young and old groups and age-related differences. *The regions where the difference between the two age groups was significant ($P < 0.05$). *Abbreviations*: FA, forearm; FH, forehead; PO, perioral area; NL, nasolabial area; SD, standard deviation.

values were significantly higher than the young group, are known to have a high appendage density (19), and the enlargement of the sebaceous glands in the elderly (13) might explain that in the older group the absorption of HN seems to be higher where the appendage density increases.

Reviews and investigative studies have been published discussing the contribution of the various structures of the skin in the drug diffusion. Some note that the contribution of the appendages in the skin permeability to chemicals should not be overlooked, especially during the early phase of absorption (20–22). The appendageal

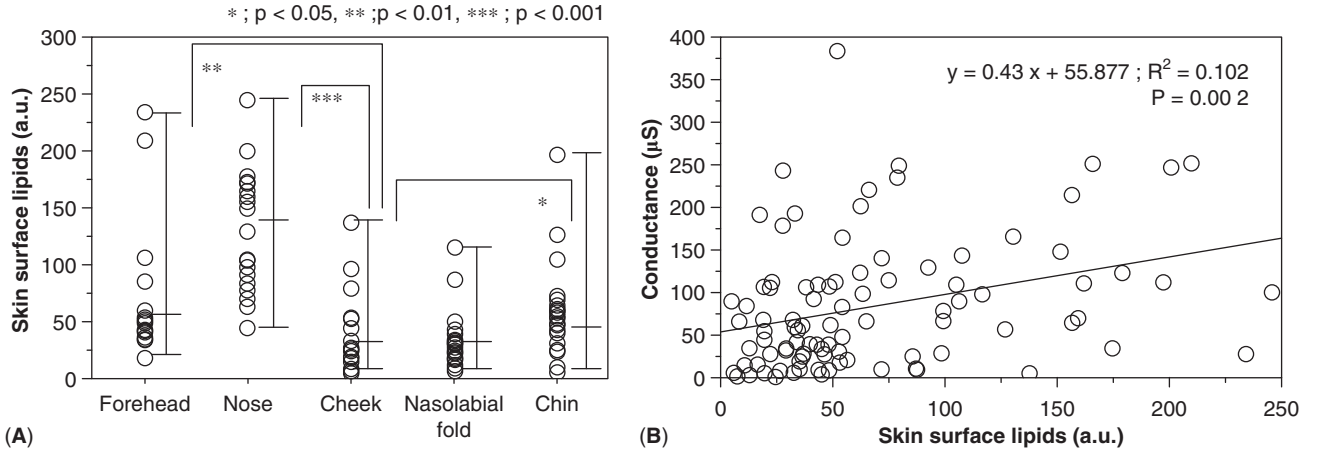

FIGURE 34.4 The skin surface lipids on the forehead, nose, cheek, nasolabial fold, and chin. The skin surface lipids on the forehead, nose, and chin were significantly higher than that on the cheek (**A**). There was a significantly positive correlation between skin surface lipids and high frequency conductance (**B**).

route was reported to contribute methyl nicotinate transport in the skin (5). Using normal artificially damaged skin (without follicles and sebaceous glands), Hueber et al. (23) demonstrated that the appendageal route accounts for the transport of hydrocortisone and testosterone, but is more important for this latter and more lipophilic compound. Illel et al. (24), studying rat skin, found that appendageal diffusion is a major pathway in the absorption of hydrocortisone, caffeine, niflumic acid, and *p*-aminobenzoic acid.

Other studies (25,26) suggest that intercellular lipid composition is a major factor in barrier function. Kobayashi and Tagami (12) found that skin surface lipids were richest on the nose. Comparatively, the skin surface lipids on the forehead, nose, cheek, nasolabial fold, and chin with the skin surface lipids on the forehead, nose, and chin significantly higher than that on the cheek (Fig. 34.4A) and there was a significantly positive correlation between the skin surface lipids and high frequency conductance (Fig. 34.4B).

Transepidermal water loss (TEWL) is a parameter for the skin barrier function used to measure the skin water barrier. TEWL is measured with instruments based on an open chamber system equipped with a thermister and humidity sensors that measure the water gradient above the skin surface produced by the water evaporating through the SC (12). Shriner and Maibach (4) investigated regional variations in the SC functions and skin reactivity to benzoic acid in different parts of the face, finding that the skin of the nasolabial area had the highest TEWL, as the most sensitive area on the face (12). Previous studies (12) indicate that TEWL increases in irritability of the skin in young subjects and shows a decrease with age and nasolabial fold and the chin showed a significant proneness to decrease with age as indicated by a study of 303 Japanese females (9).

TEWL values for the forehead, nasolabial fold, nose, chin, and cheek were measured and the results indicated that values from the forehead, nasolabial fold, nose, and chin were significantly higher than those obtained from the cheek (9). Lopez et al. (27) found that mean TEWL values on the forehead were significantly lower than on the cheek, measured by Evaporimeter EP1C. The highest mean values were obtained on the chin (12.7 ± 1.4 g/m^2/h) and lowest mean values on the forehead (9.6 ± 2.1 g/m^2/h). Moreover, other studies (18) have demonstrated that of the forehead, upper eyelid, nose, cheek, nasolabial area, perioral area, chin, neck, and volar forearms studied in individuals 24–34 years of age, nasolabial and perioral areas showed the highest TEWL values: 14.0 ± 4.4 and 15.8 ± 11.6, respectively. Differing data regarding TEWL values on the cheek versus forehead may be a result of differences in measurement sites and technique (18).

A hypothesis regarding TEWL suggests that the barrier properties of this thin layer is due to structure, which consists of pentagonal or hexagonal corneocytes embedded in a lipid matrix. Because the main route of permeation is around the corneocytes, this means that the larger the corneocytes are, the longer the route for the permeation will be (28). Corneocyte size is dependent on the site on the body and this can be directly related to the permeability, suggesting that there is a linear relationship between corneocyte size and the number of cell layers. Thus, the larger the corneocyte size is, the more layers it will have (28).

However, one should keep in mind that skin reactivity to HN is probably not only the expression of the sole transcutaneous penetration of the molecule, but also the manifestation of individual variability in the vascular response to HN.

Stratum Corneum Turnover

The SC turnover was slower in the nasolabial area and the forearm in both the age groups.

Kawaie et al. (29) reported in women a longer transit time in the volar forearm than in the face. In previous studies reviewed by Grove and Kligman (30), the SC turnover of the forearm was reported to decrease in the elderly. In our study the same trend was found although not statistically significant in all areas, probably because of the shorter application time of dansyl chloride (16 hours). Kawai et al. (29) did not find changes in the SC turnover in the face in the elderly. However, Kobayashi and Tagami (12) found that SC barrier function is increased with age due to the decreased epidermal turnover rate recognized by the increase in corneocyte size. Furthermore, the elderly have been indicated to show an increased SC barrier function (6,8) and attenuated response to certain irritants (4,10–13).

So, in the volar forearm a photoprotected area, mainly the aging process is responsible for the differences between SC turnover between the age groups. The face is the most exposed area to the

UV radiations. This could account for the reverse trend in the SC turnover, although endogenous factors could also intervene. In the face, the SC transit time was shorter than in the protected area (the forearm) and in the older subjects who have received much UV radiation during their life than the young group. So it seems that the photoaging process probably decreases the SC transit time.

CONCLUSION

Many factors certainly account for the percutaneous absorption of the drugs. Besides the various physical parameters used in our study, noninvasive methods for the study of the appendageal density (31) and the SC lipids composition (32) have been considered to evaluate the influence of these two parameters on percutaneous absorption of chemicals. Previously, noninvasive study of SC lipid composition was an influence of percutaneous absorption of chemicals. Results show that skin surface lipids richest on the nose, whereas superficial pH on the nose was lowest among the regions tested (12). This chapter highlights the necessity for further research, focusing on the standardization of anatomic sites using experimental protocols involving TEWL, sebaceous glands, lipids, and vascular changes across the face.

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35 Adhesive tape stripping reveals differences in stratum corneum cohesion between Caucasians, Blacks, and Hispanics as a function of age

Kaley A. Myer, Frank Dreher, Alessandra Pelosi, Kazuhiro Mio, Enzo Berardesca, and Howard I. Maibach

INTRODUCTION

The stratum corneum (SC) is the main barrier for skin penetration of xenobiotics (1). Its thickness in healthy human adults varies from 5 to 20 μm (2). SC can be removed by repeated application of appropriate adhesive tapes (3).

Although the influence of ethnicity (race) is today largely unknown, intrinsic properties related to skin age reportedly affect ease of SC removal (4).

Cohesion between individual corneocytes is predominantly provided by corneodesmosomes, structurally modified keratinocyte desmosomes (Egelrud et al., 1990); (5). The cohesive forces are assumed to be mediated by the corneodesmosomal transmembrane glycoproteins desmoglein and desmocolin (6–8). On maturation of the SC from the stratum compactum to the stratum disjunctum, the nonperipheral corneodesmosomes are degraded by specific proteases and glycosidases (Rawlings, 2003), resulting in corneocyte desquamation. Furthermore, barrier lipids embed the corneocytes and contribute to SC cohesion. This can be demonstrated in numerous disorders in cornification associated with primary abnormalities in lipid metabolism (9) (Williams and Elias, 1987; Williams, 1991). For instance, cholesterol sulfate was demonstrated to be involved in the regulation of desquamation, acting as a serine protease inhibitor (10) (Williams and Elias, 1981; Sato et al., 1998).

Ethnic (racial) variability in skin physiology has been minimally investigated and existing data are often contradictory (reviewed in (11–13)). Understanding ethnic (racial) differences in skin barrier function is not only important for designing customized skin care and dermatologic products, but may also help to better understand a variety of dermatologic disorders between race or ethnicities (14). This study investigates ethnic (racial) differences and age-related differences in SC cohesion by sequential tape stripping using both traditional weighing and protein assays.

MATERIALS AND METHODS

Volunteers

Caucasian, Black, and Hispanic volunteers of two different age groups with no history of chronic dermatologic disease were recruited. Caucasians were of Anglo-Saxon European origin, Blacks were African Americans and the Hispanics were Mexican-Americans, none of whom identified with indigenous cultures. All subjects had both parents and grandparents with the same ethnic (racial) identity. Volunteers of both sexes were selected as Jacobi showed no differences in SC thickness/adherence between sexes (15) and to obtain a comprehensive view of ethnic differences. Young volunteers were between 20 and 40 years, and elderly volunteers were between 60 and 85 years of age (Table 35.1). Six healthy volunteers (three males and three females) per ethnic group entered the study. Subjects were instructed not to apply any topical products to the test sites for four weeks prior to the study. The subjects rested for at least 30 minutes before the study onset. There was no pretreatment. The study was performed during summer in San Francisco (CA). The clinical study was performed in accordance with the guidelines of "Good Clinical Practice" that underwrites the principles of the Declaration of Helsinki on human experimentation. Written informed consent was obtained from each subject and ethical approval was provided by the Committee on Human Research, University of California, San Francisco.

Transepidermal Water Loss

Transepidermal water loss (TEWL) was measured prior to tape stripping and five minutes after completion of stripping using an evaporimeter (Tewameter TM210; Courage-Khazaka, Germany; Acaderm, Menlo Park, CA). A single measurement per skin site was performed. Room temperature and humidity were monitored and ranged between 19 and 22°C and between 50 and 60% relative humidity during the study.

Tape Stripping

Using gloved hands, adhesive tape stripping was performed on one site of the volar forearm in the center between cubital fossa and wrist. One test site per subject was chosen. There was no pretreatment to the area prior to stripping. The test site was outlined with a marking pen and sequentially stripped 40 times with a preweighed D-Squame disc (diameter 2.20 cm; CuDerm Corporation, Dallas,

TABLE 35.1
Age Distribution of Human Volunteers

	Caucasians		Blacks		Hispanics	
	Young	*Older*	*Young*	*Older*	*Young*	*Older*
Age range (yr)	27–37	66–81	23–34	60–85	21–36	62–82
Mean ± SD (Median)	32 ± 4 (31)	74 ± 5 (75)	32 ± 4 (34)	68 ± 2 (68)	31 ± 6 (34)	72 ± 7 (72)

Age range, mean ± SD ($n = 6$) and median as a function of ethnic (race) and age group are given.
Abbreviation: SD, standard deviation.

TX, U.S.A.) applying constant and uniform pressure (10000 Pa) for 5 seconds using a weight system. After removing the pressure, the tape was gently peeled unidirectionally from skin with forceps. After weighing, it was placed adhesive side upward into a 20 mL glass vial (Fisherbrand scintillation vials; Fisher Scientific, San Francisco, CA, U.S.A.) using forceps.

Weighing

Tapes were weighed before and after stripping using a Mettler AE163 balance (Mettler-Toledo, Switzerland) set at its highest precision of 10 μg. The balance was installed on a stone slab placed on a firm, vibration-free table to maximize damping of vibration. The weight difference between the tape before and after stripping was calculated. As has occurred in rare cases, when a negative weight difference was obtained, the amount of SC removed by this tape strip was changed to 0 μg.

Protein Assay

The protein assay was performed according to the method described by Dreher (16). One milliliter of 1 M NaOH (Fisher Scientific) was added to each vial containing a tape strip. Then, the vials were shaken for 2 hours at room temperature in order to dissolve the SC protein fraction adhering to the tape. One milliliter of HCl (Fisher Scientific, Houston, Tx) was added to the vials to neutralize the alkaline solution. Then, the protein assay was performed using the Bio-Rad Detergent Compatible (DC) protein Assay Kit following the microassay procedure (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Absorbance at a wavelength of 750 nm was measured using a Hitachi U-2001 UV–vis Spectrophotometer (Hitachi Instruments, San Jose, CA, U.S.A.). Gloves were worn to minimize protein contamination from the investigator's hands.

Stratum Corneum Standard Curve

Human cadaver skin was used to prepare SC sheets. The full-thickness skin was excised no later than 24 hours after death and stored at −20°C for a maximum of three months before use. The epidermis was obtained by heat separation (17). Excess subcutaneous fat was removed from the skin, which was then immersed in water at 60°C for 30 seconds. The epidermis was separated from the dermis and placed overnight in 0.0001% (w/v) trypsin type III (Sigma, St. Louis, MO, U.S.A.) in phosphare-buffered saline (Sigma) at 37°C. The epidermis was removed from the SC sheets; they were air-dried, quickly rinsed with ice-cold hexane (Fisher Scientific) to remove the superficial lipids adhering to the sheets and finally stored under vacuum at room temperature until use. A stock solution was prepared from a fixed mass of SC in an appropriate volume of 1 M NaOH (Fischer Scientific, Houston, Tx) and used to create a standard curve.

Surface Glistening

Surface glistening was observed visually and defined as a glossy appearance of the skin surface being tape stripped. The tape strip number, after which surface glistening was first observed, was recorded.

Statistical Analysis

Statistical analysis was performed using software SPSS version 11.5 (SPSS, Chicago, IL, U.S.A.). One-way analysis of variance completed with pair-wise multiple comparisons according to Tukey was performed to select for significance between ethnicities (races) for a given age group. The unpaired, two-sided Student's *t* test was used to select for significance between young and older subjects for a given ethnicity (race) after confirming that the data were normally distributed, using the Kolmogorov–Sminov test. The difference between the two SC quantification methods was analyzed by applying the paired, two-sided Student's *t* test. Statistical significance was accepted when $P \leq 0.05$. A trend toward a statistical significant difference was accepted when $0.05 < P \leq 0.10$. The data were presented as cumulative sums of SC mass removed after 1, 10, 20, 30, or 40 sequential tape strips.

RESULTS

Tape Stripping Reveals Ethnic Differences in Mass Removal

A larger mass of SC was removed for young Blacks as compared with young Hispanics after one tape strip ($p_{weighing} = 0.05$, $p_{protein} = 0.06$), 10 ($p_{weighing} = 0.02$, $p_{protein} = 0.03$), 20 ($p_{weighing} = 0.02$, $p_{protein} = 0.04$), 30 ($p_{weighing} = 0.04$, $p_{protein} = 0.06$), and 40 ($p_{weighing} = 0.04$, $p_{protein} = 0.07$) . The young Blacks had an increase of 188% by mass or 71% by weight compared with the Hispanics after one tape strip. This difference decreased with increasing tape strips (Figs. 35.1A and 35.2A). No race-related differences ($P > 0.10$) were found between young Caucasians and young Blacks, between young Caucasians and young Hispanics or between older Blacks and older Hispanics.

A larger, although not statistically significant, mass was removed from older compared to younger Caucasians ($p_{weighing} = 0.06$, $p_{protein} = 0.07$) as well as for older compared to younger Hispanics after 10 strips ($p_{weighing} = 0.09$, $p_{protein} = 0.06$). No age-related differences in

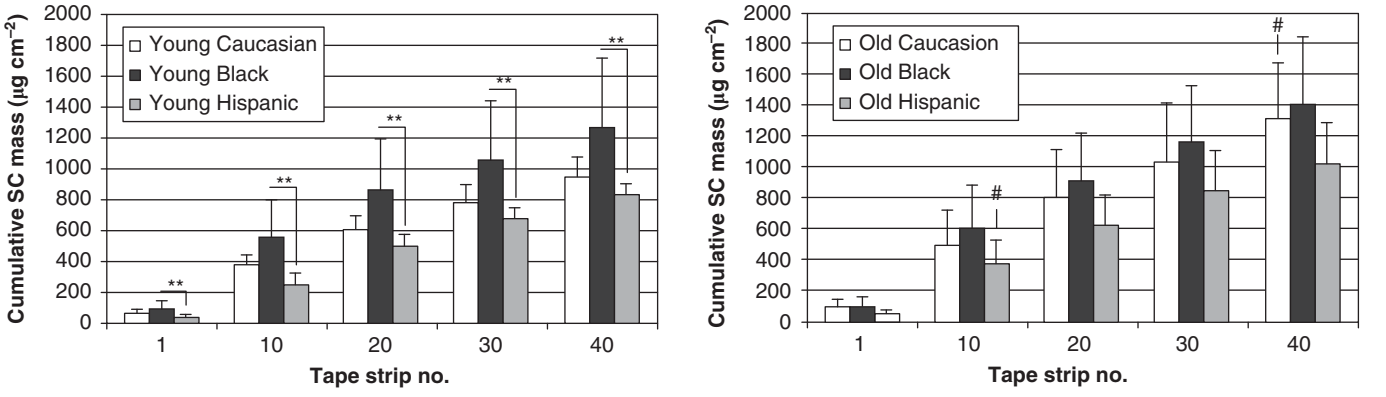

FIGURE 35.1 Weighing of the SC mass removed with repetitive adhesive tape stripping as a measure for SC cohesion revealed race-related differences on the volar forearm as a function of age. SC cohesion of Hispanics is decreased (**$P \leq 0.05$, $0.05 < {}^{*}P \leq 0.10$) compared with Blacks in (**A**) young subjects but not in (**B**) older subjects, whereas the SC of Caucasians and Blacks were shown to be of comparable, but decreased cohesiveness. Globally, SC cohesion was found to decrease with age. However, a significant or a trend toward a statistically significant decrease (##$P \leq 0.05$, $0.05 < \#P \leq 0.10$) was only found for Caucasians after 40 tape strips and for Hispanics after 10 strips. The results are represented as cumulative sums of SC mass removed per tape surface area (µg/cm^2) after 1. 10, 20, 30, or 40 sequential tape strips; the mean ± SD of six subjects per ethnic group and per age group is given. *Abbreviations*: SC, stratum corneum; SD, standard deviation.

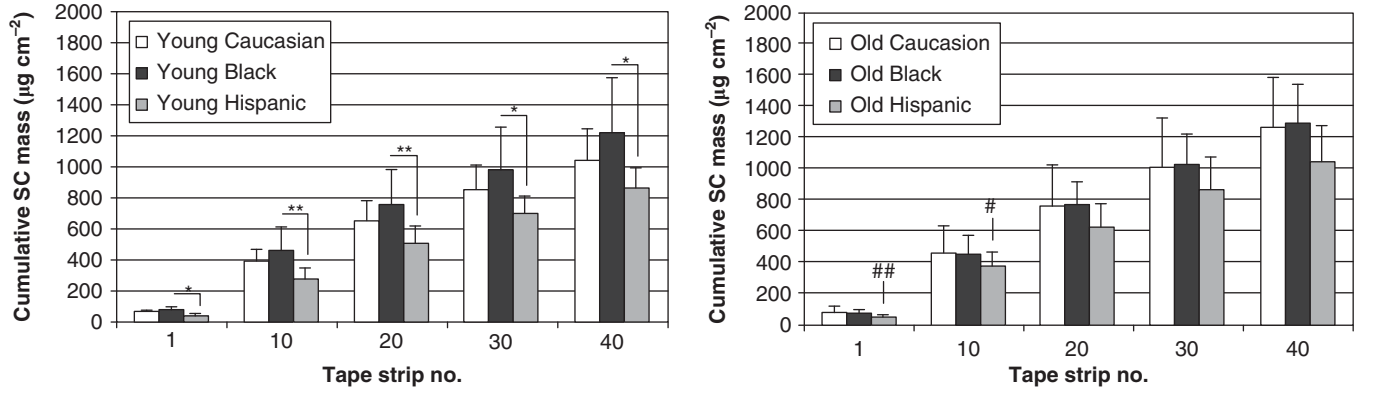

FIGURE 35.2 Quantification of total protein with repetitive adhesive tape stripping as a measure for SC cohesion revealed ethnic-related differences on the volar forearm as a function of age. SC cohesion of Hispanics is decreased (**$P \leq 0.05$, $0.05 < P \leq 0.10$) compared with Blacks in (**A**) young subjects but not in (**B**) older subjects, whereas the SC of Caucasians and Blacks were shown to be of comparable but decreased cohesiveness. Globally, SC cohesion was found to decrease with age. However, a significant or a trend toward a statistically significant decrease (*$P \leq 0.05$, $0.05 < {}^{*}P \leq 0.10$) was only found for Hispanics after 1 and 10 strips, respectively. The results are represented as cumulative sums of SC mass removed per tape surface area (µg/cm^2) after 1, 10, 20, 30, or 40 sequential tape strips using the protein assay described by Dreher and coworkers (15); the mean ± SD of six subjects per ethnic group and per age group is given. *Abbreviations*: SC, stratum corneum; SD, standard deviation.

the SC mass were found for the Caucasians before tape strip 40 and for Hispanics after tape strip 10. Furthermore, no age-related differences were observed for Blacks.

Surface Glistening

Surface glistening was observed for some subjects before removing 40 tape strips. This was observed for two young subjects (after 23 and 39 tape strips) and five older Caucasians (34–39 strips), for one young (38 strips) and one older Black (35 strips) as well as for three young (38–40 strips) and three older Hispanic subjects (33–39 strips). In these subjects, further tape strips continued to remove mass, shown by weighing and protein assay, but these data were not included in the analysis.

Transepidermal Water Loss Reveals No or Only Slight Ethnic Differences

TEWL was measured at the tape stripping site on the volar forearm before tape stripping (baseline measurement) and after completion of the 40 strips. The respective TEWL difference was calculated. The results are in Table 35.2 as a function of ethnicity and age. Neither baseline TEWL between ethnic-(race-)matched young and ethnic (race-)matched elderly subjects, nor baseline TEWL between age-matched Caucasians, Blacks, and Hispanics were statistically different ($P > 0.05$). However, there was a trend (although not statistically significant, $P = 0.06$) toward a higher baseline TEWL for young Caucasians compared with young Black subjects. Furthermore, no differences were found between age-matched ethnic groups as well as ethnic-matched age groups for TEWL measured poststripping and TEWL difference, respectively.

Weighing is Less Accurate to Quantify SC than Protein Assay

To illustrate the pattern of SC mass determined by both the quantification methods as a function of tape strip number, a representative example of the entire volunteer panel is shown in Fig 35.3A. Although the tape stripping procedure was standardized with

TABLE 35.2
Sequential Tape Stripping Did Not Reveal Any Ethnic-(Race-)Related Differences in SC Integrity

	Caucasians		Blacks		Hispanics	
	Young	*Older*	*Young*	*Older*	*Young*	*Older*
Before tape stripping (Median)	6.8 ± 1.3* (6.3)	7.4 ± 4.0 (5.5)	5.0 ± 1.4* (5.0)	4.9 ± 0.8 (4.8)	6.7 ± 1.1 (6.7)	5.4 ± 1.7 (5.9)
After tape stripping (Median)	23.4 ± 18.0 (15.4)	34.7 ± 19.1 (42.2)	18.8 ± 10.4 (16.6)	21.1 ± 14.1 (19.7)	32.1 ± 11.4 (22.2)	19.8 ± 17.4 (11.9)
ΔTEWL (Median)	16.6 ± 17.0 (9.1)	27.2 ± 18.2 (32.1)	18.8 ± 9.8 (11.0)	19.3 ± 14.3 (14.9)	16.4 ± 11.6 (14.9)	14.4 ± 16.3 (6.0)

SC integrity was assessed by TEWL (g/m²/h) before tape stripping and after completing 40 tape strips. TEWL after tape stripping as well as the respective TEWL difference (ΔTEWL) reflected the influence of tape stripping on SC integrity and were not different ($P > 0.10$) between age-matched ethnic groups or ethnic-matched age groups. Besides a trend toward a higher TEWL for young Caucasians as compared with young blacks ($0.05 < *P \leq 0.10$). There were not different baseline TEWL observed between ethnic groups.

Abbreviations: SC, stratum corneum; SD, standard deviation; TEWL, transepidermal water loss.

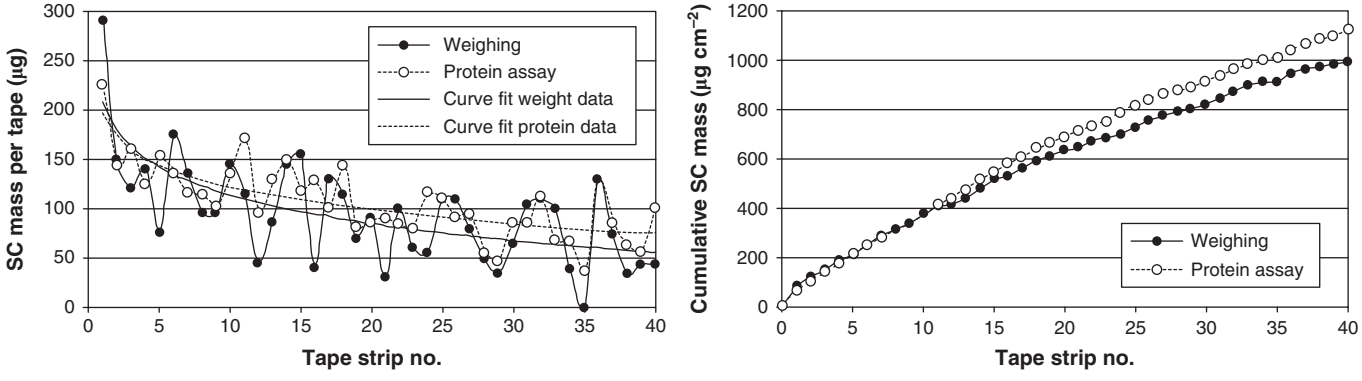

FIGURE 35.3 Weighing to quantify SC amount removed by adhesive tape stripping generally provided more variable and less accurate mass data compared with the protein assay. A representative example of the typical pattern of the SC mass removed and quantified by weighing and by protein assay as described by Dreher et al. (15) is shown. (**A**) Independent of the quantification method and for all subjects enrolled in this study, larger amounts of SC (µg) were removed during the initial first strips, whereas decreasing amounts were removed with increasing strips. (**B**) Similar results were obtained for cumulative sums of SC mass per tape surface area (µg/cm²) up to 40 sequential tape strips, although weighing provided more variable data than the protein assay. *Abbreviation*: SC, stratum corneum.

respect to application and removal, often seemingly inconsistent amounts of SC were removed between a tape strip and the immediately succeeding one. This inconsistency seems to be more prominent when the tapes were weighed than the protein assay. And, independent of race and age, a pronounced oscillation-like pattern in the function between tape strip number and SC mass determined by weighing was observed as depicted in Fig 35.3A in about one third of the subjects. Furthermore, in 1.3% of measurements by weighing there was no mass difference between the tape before and after stripping. Similar behavior was observed when comparing cumulative SC mass removal. The variability between individual data points could be quantitatively expressed by the regression coefficient R^2 of the natural logarithmic curve fit 1 of the function tape strip number *versus* SC mass removed.

$$\text{SC [µg]} = a^*\ln(\text{number of tape strips}) + b$$

The logarithmic function 1 was chosen to describe the "exponential-like" function of tape strip number *versus* SC mass removed after performing an analysis of the residuals, which approximated random errors. The regression coefficient R^2 of the curve fit for all subjects enrolled in this present study was significantly higher ($P = 0.001$) when the SC quantification was performed by the protein assay (mean ± SD = 0.41 ± 0.25, median = 0.37). Coefficient *a* represents the "slope" of the logarithmic function 1 and was negative for all volunteers. Coefficient *b* reflects the (positive) "intercept" and is a direct measure of the SC mass removed after the first strip.

The SC mass determined by weighing moderately correlated ($R^2 = 0.71$) with the mass determined by the protein assay when each individual tape strip (total 1440) was included in the linear regression analysis. However, statistically significant higher ($P < 0.05$) or a tendency toward higher ($0.05 \leq P < 0.10$) SC amounts were obtained when performing the SC quantification by weighing compared with the protein assay for elderly Caucasians ($P = 0.03$) and for elderly Blacks ($P = 0.06$) after one tape strip, as well as for young Hispanics after 10 tape strips ($P = 0.06$).

DISCUSSION

Ethnic (racial) differences in skin barrier function have been minimally investigated and contradictory results reported (reviewed in (11–13,18). The present study aimed, therefore, to further elucidate ethnic differences in SC properties by adhesive tape stripping.

The present study demonstrated that tape stripping removes significantly larger quantities of SC for young Black subjects than young

Hispanics. Yet no such race-related differences were found between elderly Blacks and elderly Hispanics. Moreover, tape stripping did not reveal any race-related differences in skin physiology between Caucasians and Blacks or between Caucasians and Hispanics independent of their age. Larger quantities of SC were removed in older subjects than younger ones for Caucasians and Hispanics. However, no age-related differences were observed for Blacks in this study.

Since SC cohesion is provided by corneodesmosomes, differences in their frequency, distribution, composition, and structure might be associated with the observed differences in SC cohesion between some ethnic (race) and age groups. Yet, to the best of our knowledge, there are no study reports on ethnic and age-related differences in corneodesmosome characteristics. Differences in the corneodesmosome degradation process leading to subsequent corneocyte desquamation might also influence SC cohesion. The few studies existing on racial differences in corneocyte desquamation or skin scaliness (19,20), are, however, inconclusive. For instance, a significantly increased spontaneous corneocyte desquamation on the upper arm of young Black subjects than young Caucasians was reported (21). However, no difference in the scaliness was found between Caucasians and Blacks at different anatomic sites, including the volar forearm, except the face for age-matched subjects (20,22). In a similar study design, we confirmed these latter observations for the volar forearm by quantifying SC removed with the initial tape strip instead of measuring scaliness. However, whether the mass of SC removed with the first tape strip and skin scaliness evaluated by image analysis are indeed related, requires a more detailed investigation. Similarly, how spontaneous corneocyte desquamation correlates to skin scaliness and/or mass of SC removed with the first tape strip is minimally known. For example, Leveque et al. reported on the evolution of SC cohesion assessed by tape stripping and spontaneous corneocyte desquamation on the volar forearm as a function of age (4). Congruent with our results, they obtained an increased quantity of SC stripped for elderly Caucasians and increased spontaneous desquamation with age. They interpreted the results as a greater accumulation of corneocytes in the *stratum disjunctum* of elderly persons, which accounts for the dryness and roughness of the skin surface.

Further research is needed to determine the etiology of differences in ethnicities. Possible explanations include differences in skin hydration and pH. For instance, dry atmospheric conditions have been shown to decrease the water content of murine SC, leading to impaired skin desquamation and induction of scaly skin (23). Similarly, an increased quantity of SC is removed by tape stripping for elderly Caucasians (4), this study), whose SC is less hydrated than young Caucasians (24–26). The fact that there was no significant age-related difference for Blacks, although their skin was also reported to be less hydrated with age (20), might be explained by the larger variability of data for young Blacks than young Caucasians in the present study. Because the existing water hydration data and skin pH data are limited and rather inconclusive for Hispanics, it seems difficult to interpret the altered SC cohesion compared with young Blacks observed in the present study in terms of differing cutaneous water content or pH.

Because skin conductance and capacitance are not based solely on the SC hydration, but on sweat production or the presence of hair on the measuring site as well (reviewed in (27,28), racial differences in skin hydration should be interpreted with caution.

It was demonstrated that more cellophane tape strips were needed to completely remove occluded skin of Blacks than Caucasians after presoaking skin with water for 24 hours under occlusion (29). As earlier studies indicated no differences in SC thickness between Caucasians and Blacks (30,31) these data imply a greater intercellular SC cohesion for hydrated skin in Blacks than Caucasians. However, we did not find that normally hydrated Caucasian and Black skin differs in intercellular SC cohesion. This may be because our experiment did not have the power to show an existing difference, or because hydrating the subjects' skin prior to the experiment improved the hydration status of our subjects' skin. Caucasian subjects are more prone to dry skin, which can lead to an increased perception of scaliness (32,33).

Other than differences in corneodesmosome physiology, differences in skin barrier lipids may further explain racial differences in SC cohesion of normal skin. Although no peer-reviewed data exist on this topic yet, one study showed a significant reduction in ceramide, cholesterol, and fatty acid levels with age for female Caucasians, whereas the ratio of the different lipids remains constant (34).

We did not find any significant baseline TEWL differences on the volar forearm between young and older Caucasians, Blacks, and Hispanics, respectively. Earlier studies (35) confirmed these results for Caucasians, but they are in contrast to other studies reporting higher TEWL for young subjects compared with older ones (4,36,37). This discrepancy in TEWL data between age groups may be explained by the small sample size of our study. To the best of our knowledge, age-related TEWL differences for other ethnic groups than Caucasians were never reported. Furthermore, the fact that we could not reveal any race-related differences in AC integrity after performing sequential tape stripping—although differing amounts of SC and thus possibly also different fractions of the entire SC were removed between ethnic groups (races)—may be explained by the enormous variability in TEWL measured poststripping.

As demonstrated, the total protein assay to quantify SC mass removed by tape stripping provided an accurate method to investigate SC cohesion. Weighing often resulted in more variable mass data points within one series of repetitive tape strips compared with the protein assay. And, weighing became sometimes even impossible, since negative or no weight differences were obtained, whereas protein could still be largely quantified on the tape. This can be explained by the fact that the precision of weighing (with an error of ±20 μg) is insufficient for accurately quantifying small masses of SC per tape strip (<100 μg). Such amounts were frequently removed after performing 20–30 sequential tape strips under the present conditions. In some cases, nonetheless, the SC mass determined by the protein assay was significantly smaller than weighing. This was observed most with the first tape strips in elderly subjects. Several possible reasons can be indicated to explain this divergence: (*i*) the extraction of SC adhering to the tape was not complete under the present protein assay conditions when huge SC quantities were removed, and (*ii*) the initial tape strips contain sebum or other nonproteinaceous material, which was not taken into account by the protein assay but by weighing instead.

In summary, the present study convincingly demonstrated that adhesive tape stripping combined with accurate SC quantification reveals variation in SC cohesion between ethnic groups. Whereas the SC of young Hispanics seems particularly cohesive, the SC of Caucasians and Blacks belonging to the same age group, were shown to be of comparable, but decreased cohesiveness.

Age-related differences in SC cohesion were also determined showing globally a diminished SC cohesion with age independent of race. Further ultrastructural investigations in corneodesmosome physiology in combination with cutaneous biometrics measurements should therefore help to further elucidate such differences in SC cohesion.

Taken together, these data show much additional information and shows the need for more information before we understand these racial differences.

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36 Animal, human, and in vitro test methods for predicting skin irritation

Yakir S. Levin, Cheryl L. Levin, and Howard I. Maibach

INTRODUCTION

Contact with external irritating agents, such as dishwashing liquid, enzymes, or raw meat, can result in irritant contact dermatitis (ICD), a localized condition associated with the innate immune system. ICD ensues when irritant stimuli overpower the defense and repair capacities of the skin (1). Exposure to potent irritants or exposure to mild irritants for an extended period of time will increase the likelihood of developing ICD. Preventive measures, including the utilization of proper skin care, the avoidance of harsh soaps, and the use of protective garments, such as gloves, will decrease the risk of irritant dermatitis occurring. In addition, it is of crucial importance to test the irritant potential of any substance that will be applied to human skin, so that its likelihood of inducing irritant dermatitis is known. Federal regulatory agencies require toxicity testing to determine the safety or hazard of various chemicals and products prior to human exposure. This information is used to properly classify and label products according to their potential hazard (2). No one assay is able to accurately portray irritation in its entirety. This is because irritant dermatitis may result from either acute or cumulative injury, and may involve inflammation or skin necrosis (corrosive). A number of animal, human, and in vitro test methods have been developed, each portraying some but not all aspects of irritation. Each model has its unique benefits and limitations.

ANIMAL MODELS

Draize Rabbit Assay

To evaluate primary irritation and corrosion, the Draize animal model or one of its modifications is used. The Draize rabbit test was developed in 1944, and has since been adopted in the U.S. Federal Hazardous Substance Act (FHSA) (code of FR) (3). The test involves two (1 in.^{2}) test sites on the dorsal skin of six albino rabbits. One site is abraded (through the use of a hypodermic needle across the rabbit skin) and the other site remains intact. The stratum corneum is broken on the abraded site, without loss of blood. The undiluted "irritant" materials (0.5 g for solids or 0.5 mL for liquids) are placed on a patch and applied to the test sites. They are secured with two layers of surgical gauze (1 in.^{2}) and tape. The animal was wrapped in cloth so that the patches were secure for a 24-hour period. The assessment of erythema and edema, using the scale noted in Table 36.1, takes place 24 and 72 hours after patch application. Severe reactions are again assessed on day 7 or 14. Radiolabeled tracers or biochemical techniques to monitor skin healing is also used by some investigators. Other investigators supplement with histologic evaluation of skin tissue (4). The Draize test ultimately quantifies irritation with the primary irritation index (PII), which averages the erythema and edema scores of each test site and then adds the averages together. Materials producing a PII of <2 are considered nonirritating, 2–5 mildly irritating, and >5 severely irritating, requiring precautionary labeling. Subsequent studies have demonstrated that the PII is somewhat subjective because the scoring of erythema and edema require clinical judgment (5). Main critics of the Draize test oppose the harsh treatment of animals. They argue that the Draize test is unreliable in distinguishing between mild and moderate irritants. Furthermore, they believe that the Draize is not an accurate predictor of skin irritancy as it does not include vesiculation, severe eschar formation, or ulceration in evaluating the PII. Finally, they argue that the Draize procedure is not reproducible (6), and they question its relevance with regard to human experience (7–9). Proponents of the Draize test point out that the test is somewhat inaccurate but it generally overpredicts the severity of skin damage produced by chemicals, and thereby errs on the side of safety for the consumer (10).

Modified Draize Models

The Draize test has been modified in response to harsh criticism. Alterations included changing the preferred species, use of fewer animals, testing on only intact skin and reduction of the exposure

TABLE 36.1
Draize Scoring System

Erythema	
No erythema	0
Slight erythema	1
Well-defined erythema	2
Moderate or severe erythema	3
Severe erythema or slight eschar formation (injuries in depth)	4
Edema	
No edema	0
Very slight edema	1
Slight edema (well-defined edges)	2
Moderate edema (raised > 1 mm)	3
Severe edema (raised > 1 mm and extending beyond the area of exposure)	4

Source: From Ref. 47.

period to irritants. Please note Table 36.2 for a comparison of the modified Draize tests.

Cumulative Irritation Assays

Frequently, ICD is produced through cumulative exposure to a weak irritant. While the Draize assay assesses acute exposure to a strong irritant, there have been many assays developed to measure repetitive, cumulative irritation. One such assay was developed by Justice et al. (11). They measured epidermal erosion through a repeat animal patch test for comparing irritant potential of surfactants. In their study, solutions were occlusively applied to the clipped dorsum of albino mice for a 10-min interval. The process was repeated seven times and the skin was subsequently examined microscopically for epidermal erosion. The repetitive irritation test (RIT), as described by Frosch et al. (12), uses guinea pigs as the animal model in determining the protective efficacy of creams against various chemical irritants. In one study, the irritants sodium hydroxide (NaOH), sodium lauryl sulfate (SLS), and toluene were administered daily for two weeks to shaved dorsal skin of guinea pigs. Barrier creams were applied 2 hours prior to and immediately after irritant exposure. Visual scoring, laser Doppler flowmetry (LDF), and transepidermal water loss (TEWL) quantified resultant erythema. The study found one barrier cream effective against SLS and toluene injury, whereas another barrier cream studied did not show any efficacy. In general, the RIT is most useful in evaluating the efficacy of barrier creams in preventing cumulative irritation. To rank products for their irritant potential, repeat application patch tests have been developed. Diluted potential irritants are occlusively applied to the same site for 15–21 days.

Of occlusion and the type of patch used to apply the irritants. In general, a longer occlusive period will result in enhanced percutaneous penetration. Similarly, the Draize-type gauze dressing will produce less percutaneous penetration as compared with the Duhring metal chambers. To facilitate interpretation of test results, a reference material that is of similar use or which produces a known effect is incorporated into the test. Rabbits and guinea pigs are the most commonly used animal species in the repeat application test (13,14). In another study, Kobayashi et al. (15) studied the effects of propranolol as an irritant using both primary and cumulative irritation assays. In both assays, skin irritation and histopathologic changes were observed in all guinea pigs treated with propranolol, and those tended to increase with the increase of propranolol dosage. The skin reactions increased with the application times of propranolol up to seven days in the cumulative skin irritation study. Scoring of the test sites were made in accordance with the following scale: 0 = no reaction, 1+ = mild erythema covering the entire patch area, 2+ = erythema and edema, 3+ = erythema, edema, and vesicles, 4+ = erythema, edema, and bullae. One variation of the repeat application patch test involves measuring the edema-producing capacity of irritants using a guinea pig model. Visual inspection and Harpenden calipers measure skin thickness after the application of irritants for 3–21 days. This model demonstrates clear dose–response relationships and discriminating power for all irritants, excluding acids and alkalis (14). Open application assays, developed by Marzulli and Maibach (16), involve application of irritants onto the backs of rabbits 16 times over a three-week period. Visual scoring of erythema and skin thickness measurements were used to quantify results. A high degree of correlation has been observed when comparing erythema and skin thickness data. In addition, the results of 60 test substances in rabbits strongly correlated with the results of cumulative irritation studies in man, suggesting that the rabbit assay is a useful model. A modified open application assay was performed by Anderson et al. (17). In his assay, irritants are applied once a day for three days to a 1 cm^2 test site on the backs of guinea pigs. The sites were evaluated visually for erythema and edema. In addition, biopsies were taken and skin samples were stained with May–Grunward–Giemsa under oil immersion, to evaluate epidermal thickness and dermal infiltration. Irritants were compared with the standard irritant, 2% SLS, and their potency was ranked. Extensive processing involved in properly performing this assay may limit its usefulness.

TABLE 36.2
Modified Draize Irritation Method

	Draize	FHSA	FIFRA	DOT	OECD
No. of animals	3	6	6	6	6
Abrasion	Yes	Yes	2 of each	No	No
Exposure period (hr)	24	24	4	4	4
Examination (hr)	24, 72	24, 72	0.5, 1, 24, 48, 72	4, 48	0.5, 1, 24, 48, 72
Excluded from testing	–	–	Toxic material pH 2 or 11.5	–	Toxic material pH 2 or 11.5

Abbreviations: FHSA, Federal Hazardous Substance Act; FIFRA, Federal Insecticide, Fungicide and Rodenticide Act; DOT, Department of Transportation; OECD, Organization for Economic Cooperation and Development.
Source: From Ref. 2.

Immersion Assay

Aqueous detergent solutions and other surfactant-based products are evaluated for irritancy using the guinea pig immersion assay (18–20). This assay involves placing 10 guinea pigs in a restraining device that is immersed in a 40°C test solution for 4 hours daily for a total of three days (21). The restraining apparatus allows the guinea pig's head to be above the solution. Twenty-four hours after the final immersion, the animals' flanks are shaved and evaluated for erythema, edema, and fissures. In one study, the dermatotoxic effects of detergents in guinea pigs and humans were concomitantly tested (19). The immersion assay was used to test guinea pigs, whereas the patch assay tested humans. Irritation of guinea pig skin led to epidermal erosion and a 40–60% increase in histamine content. Seven of eight human subjects had a positive patch test to the same irritants, indicating a strong correlation between the guinea pig and human models.

Mouse Ear Model

The mouse ear model is used to evaluate the degree of inflammation associated with shampoos or surfactant-based products. Uttley and Van Abbe (22) first described the mouse model when they applied undiluted shampoos to one ear of mice daily for four days. They visually assessed the erythema, vessel dilation, and edema. However, the anesthetic used to anesthetize the mice in

this study may have altered the development of inflammation and confounded results. Patrick and Maibach (3) applied surfactants to measure mouse ear thickness at various time points after the irritant application. Pretreating the ear with croton oil or 12-*O*-tetradecanoylphorbol 13-acetate 72 hours before the irritant application increased the sensitivity of the assay. This assay was most useful in testing surfactant-based products and had little efficacy with oily or highly perfumed materials.

Further animal assays were developed to quantify irritant response. Humphrey et al. (23) measured Evans blue dye recovered from rat skin after exposing the skin to inflammatory agents. Trush et al. (24) assessed the dermal inflammatory response to numerous irritants by measuring the level of myeloperoxidase enzyme in polymorphonuclear leukocytes in young CD-1 mice.

Conclusion

Animal assays must be interpreted with caution. Dose–response measurements must be followed. Draize scores are most accurate when compared with related compounds with a record of human exposure. It is important to note that occlusive application does not enhance percutaneous penetration for all materials. Responses in animal models, particularly the guinea pig and the rabbit, have a high degree of correlation to those of humans, but some inconsistencies have occurred. Major discrepancies in irritant response between different animal species tested under identical conditions have occurred (25,26), particularly with regard to weak irritants and colored materials. Subjective visual scoring techniques have accounted for some of these discrepancies. It is prudent to use other methodologies in addition to the animal model when evaluating a putative irritant.

IN VITRO ASSAYS

In vitro skin irritation assays are of potential benefit in addressing ethical concerns associated with animal testing. Additionally, there are legal barriers to the use of animals, such as the EU directive on the protection of animals used for experimental purposes (Directive 86/609/EEC), which banned the use of animal experimentation when a scientifically approved alternative exists (27).

These "alternative" methods may potentially reduce the number of animals needed in irritation testing, or in some cases may fully replace the need to use animals. A number of in vitro skin irritation assays have been developed. However, most of these have not been evaluated in validation studies to determine their usefulness, limitations, and compliance with regulatory testing requirements. Furthermore, dose–response relationships have not been established for in vitro methods. Studies evaluating in vitro testing thus far indicate usefulness in predicting starting doses for in vivo studies, potentially reducing the number of animals used for such determinations. Additionally, other studies suggest an association between in vitro cytotoxicity and human lethal blood concentrations.

Single Cell Assay

A single cell type can be cultured in plastic dishes under submerged culture conditions. Immortalized cell lines are usually used, permitting unlimited amplification of cells derived from a single source and eliminating donor variation. This allows high-throughput screening of potential irritant compounds and is therefore an ideal model system for initial industrial screening. Molecular events can be investigated, such as signal transduction pathways and cytokine/chemokine secretion upon irritant-mediated damage to the cell membrane. Such a model system, however, does not assess penetration of stratum corneum because it does not include the differentiated components of skin (28).

Epidermal Equivalent

Human epidermal equivalents consist of fully differentiated reconstructed human epidermis. In basic epidermal equivalent models primary keratinocytes isolated from routine surgical procedures are cultured while exposed to the air from above while receiving nutrients from the culture medium below. Complete epidermal differentiation occurs, resulting in basal, spinous, and granular living cell layers as well as a compact stratum corneum (29,30). The cultures therefore have a barrier function and test substances can be applied topically in a similar manner to patch testing human volunteers. Although the stratum corneum of epidermal equivalents contains all the major lipid classes found in human stratum corneum, there are quantitative differences in their distribution (29,30). Current epidermal equivalent models exhibit only a partial barrier to the passage of topically applied substances in comparison with human skin; the permeability constants for the equivalents are higher than for human skin (31). Epidermal equivalent models have been made more complex with the introduction of Langerhans cells or melanocytes into the epidermis (32,33).

The U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the U.S. National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods (NICEATM) were established to evaluate in vitro irritant testing. Two of the irritation assays approved by these bodies are epidermal equivalents, namely, EpiDerm™ and EPISKIN™. EpiDerm (EPI-200) is a three-dimensional human skin model that uses cell viability as a measure of corrosivity. It has been used with several common tests of cytotoxicity and irritancy, including MTT, IL-1a, PGE2, LDH, and sodium fluorescein permeability. EPISKIN is a three-dimensional human skin model comprised of a reconstructed epidermis and a functional stratum corneum. In a study supported by the European Center for the Validation of Alternative Methods (ECVAM), EPISKIN was useful in testing all types of potential irritants, including organic acids, organic bases, neutral organics, inorganic acids, inorganic bases, inorganic salts, electrophiles, phenols, and soaps/surfactants. With both EPISKIN and EpiDerm, the test material is topically applied to the skin for up to 4 hours with subsequent assessment of the effects on cell viability. Other commercially available epidermal equivalents that are used in validation studies include RHE (SkinEthic Laboratories) and EST-1000 (CellSystems) (34).

Skin Equivalent

Skin equivalents consist of an epidermal compartment and a dermal compartment. A reconstructed epidermis (using keratinocytes isolated from routine surgical procedures as described above) is grown on a fibroblast-populated dermal matrix in an air-exposed manner. Fibroblasts differentiate, synthesize extracellular matrix, and exhibit contractile properties. The two viable interacting cell populations (keratinocytes and fibroblasts) have been shown to possess properties

that more closely approximate their in vivo counterparts than do keratinocytes and fibroblasts cultures apart and grown as monolayers (35,36). Commercially available skin equivalents include Phenion Full Thickness Skin Model (Phenion, Dusseldorf, Germany), Apligraf (Organogenesis Inc., Cambridge, MA, USA), and AST-2000 (CellSystems, St. Katharinen, Germany).

Excised Skin

Freshly excised skin is an advanced in vitro model and is superior to current skin equivalent models as it possesses a more in vivo-like barrier competency (31), and penetration into viable layers of skin is the ultimate determinant of whether a chemical has irritant properties (37). Excised skin also contains more cell types than tissue-engineered models (including fibroblasts, endothelial cells in addition to melanocytes, Langerhans cells, and keratinocytes). The major limitation on the use of excised skin for research purposes and industrial screening is the lack of availability of large amounts of fresh human skin to be transported directly to tissue culture laboratories.

Excised animal skin enables the use of fresh skin in greater quantities for testing. Although such an approach necessitates the use of animals and therefore does not alleviate all ethical concerns, it does not involve experimentation on living animals and may reduce the number of animals used in experimentation. The Rat Skin Transcutaneous Electrical Resistance Assay is one of the four assays approved by ICCVAM and NICEATM for irritant testing. In this method, skin disks taken from the pelts of humanely killed young rats are used. When the skin barrier is compromised, there is significantly lower inherent transcutaneous electrical resistance.

Synthetic

The fourth assay approved by ICCVAM and NICEATM for irritant testing is Corrositex, a synthetic collagen matrix serving as a skin model. Corrositex elicits a color change in the underlying liquid chemical detection system (CDS). Corrositex is currently used by the U.S. Department of Transportation (U.S. DOT) to assign categories of corrosivity for labeling purposes according to the United Nations guidelines. However, its use is limited to specific chemical classes, including acids, acid derivatives, acylhalides, alkylamines and polyalkylamines, bases, cholorosilanes, metal halides, and oxyhalides. A peer review panel of NICEATM and ICCVAM elucidated some of the advantages to Corrositex, including its possible usefulness in replacing or reducing the number of animals required. Positive test results often eliminate the need for animal testing. When further animal testing is necessary, often only one animal is required to confirm a corrosive chemical. The panel also concluded that most of the chemicals identified as negative by Corrositex or nonqualifying in the detection system are unlikely to be corrosive when tested on animals for irritation potential. Nonetheless, a negative result using Corrositex still necessitates testing using other methods, such as the in vitro assays described above or direct animal experimentation.

HUMAN MODELS

After the development of the patch test, Draize et al. suggested a 24-h single-application patch test in humans. Human testing facilitates extrapolation of data to the clinical setting. Many variations of the single-application test have been developed. Testing is often performed on undiseased skin (38) of the dorsal upper arm or back. The required test area is small and up to 10 materials may be tested simultaneously and compared. A reference irritant substance is often included to account for variability in test responses. In general, screening of new materials involves open application on the back or dorsal upper arm for a short amount of time (30 minutes to 1 hour) to minimize potential adverse events in subjects.

Single-Application Patch Testing

The National Academy of Sciences (National Academy of Sciences and Committee for the Revision of NAS Publication 1138, 1977) recommended a 4-hour single-application patch-test protocol for routine testing of skin irritation in humans. In general, patches are occluded onto the dorsal upper arm or back skin of patients. The degree of occlusion varies according to the type of occlusive device; the Hilltop or Duhring chambers or an occlusive tape will enhance percutaneous penetration as compared with a nonocclusive tape or cotton bandage (10). Potentially volatile materials should always be tested with a nonocclusive tape. Exposure time to the putative irritant varies greatly, and is often customized by the investigator. Volatile chemicals are generally applied for 30 minutes to 1 hour, whereas some chemicals have been applied for more than 24 hours. After patch removal, skin is rinsed with water to remove residue. Skin responses are evaluated 30 minutes to 1 hour after patch removal to allow hydration and pressure effects of the patch to subside. Another evaluation is performed 24 hours after patch removal. The animal Draize scale is used to analyze test results (Table 36.1). The Draize scale does not include papular, vesicular, or bullous responses; other scales have been developed to address these needs. Single-application patch tests generally heal within one week. Depigmentation at the test site results in some subjects.

Cumulative Irritation Test

Using statistical analysis of test data, Kligman and Wooding (39) calculated the time to produce irritation in 50% of subjects and the dose required to produce irritation in 50% of subjects after a 24-hour exposure). Their work formed the basis for the 21-day cumulative irritation assay. The “21-day assay” is used to screen new formulas prior to marketing. The original assay involved application of a 1-in. (2.5 cm) square of Webril saturated with the test material (either liquid or 0.5 g of viscous substance) to the skin of the undamaged upper back. Occlusive tape secured the patch. Twenty-four hours after patch application, the test site is examined and the patch is reapplied. The test is repeated for 21 days. Two modifications of the cumulative irritation test were studied by Wigger-Alberti et al. (40). One assay involved Finn chamber application of metal-working fluids onto the midback of volunteers for one day. The sites were evaluated and the fluids were then reapplied for an additional two days. In the other assay, a two-week, 6-hr/day repetitive irritation test (excluding weekends) was used. Better discrimination of irritancy and shorter duration was observed with the three-day model.

Chamber Scarification Test

The chamber scarification test assesses the irritancy potential of materials on damaged skin (41,42). Subjects included in this assay

are highly sensitive to 24 hour exposure to 5% SLS (they form vesicles, severe erythema, and edema postapplication). Six to eight 10 mm^2 areas on the volar forearms are scratched eight times with a 30-gauge needle. Scarification damages the epidermal layer without drawing blood. Four scratches are parallel and the other four are perpendicular to the test site. A 0.1 g of test material (or 0.1 mL of liquid) is then applied to the scarified area for 24 hours through Duhring chambers. Nonocclusive tape is used to secure the chambers in place. With fresh specimens, patches are applied daily for three days. A visual scoring scale is used to quantify test results 30 minutes after patch removal. An analogous area of intact skin must be scored as well, so that evaluation is based on comparison between compromised and intact skin. The visual score of scarified test sites divided by the score of intact test sites, known as the scarification index, allows this comparison to be made. The relationship of this assay to prediction of irritant response from routine use has yet to be established.

Immersion Tests

Patch tests often overpredict the irritant potential of some materials. Immersion tests were established to improve irritancy prediction by mimicking consumer use. Kooyman and Snyder (21) developed the arm immersion technique to compare the relative irritancy of two soap or detergent products. Soap solutions of up to 3% are prepared in troughs and subjects immersed one hand and forearm in each trough, comparing different products or concentrations. Temperature is maintained at 41°C (105°F). The exposure period varies between 10 and 15 minutes a day for a total of five days or until observable irritation is produced on both arms. The antecubital fossa is generally the first area to experience irritation, followed by the hands (11,21). Variations on the arm immersion technique were developed so that the antecubital fossa and the hands can be tested separately. Variations incorporate different dosing regimens or measuring different endpoints. Clarys et al. (43) and Clarys and Barel (44) investigated the effects of temperature and anionic character on the degree of irritation caused by detergents. TEWL, erythema (colorimetry, a* parameter), and skin dryness (capacitance) were used to quantify test results. The irritant response was increased by higher temperature and higher anionic content. Using a modified arm immersion technique, Allenby et al. (45) noticed that once skin had been compromised (erythema of 1+ on a visual scale), irritants applied to the forearm and back caused an exaggerated response.

Soap Chamber Technique

The "chapping" potential of bar soaps is evaluated with the soap chamber technique, developed by Frosch and Kligman (42). While patch testing is useful in predicting erythema, it does not address the dryness, flaking, and fissuring observed with bar soap use. Using this method, 0.1 mL of an 8% soap solution is applied to the forearm through Duhring chambers fitted with Webril pads. Nonocclusive tape is used to secure the chambers. Patches are applied for 24 hours on day 1 and 6 hours on days 2–5. If severe erythema at the test site occurs, the investigator must discontinue the study. Skin responses are evaluated with visual scoring of erythema, scaling, and fissures. This test correlates well with skin-washing procedures but tends to overpredict irritant response of some materials.

Protective Barrier Assessment

The skin barrier function assays test the efficacy of protective creams in preventing an irritant response. Zhai et al. (46) studied the effect of barrier creams in reducing erythema, edema, vesiculation, and maceration. Subjects were given creams and then irritated with either SLS or ammonium hydroxide. Paraffin wax in acetyl alcohol was the most effective in preventing irritation. In another study by Wigger-Alberti and Elsner (40), petrolatum was applied to the backs of 20 subjects. Subjects were then exposed to SLS, NaOH, toluene, and lactic acid. Irritation was assessed by visual scoring, TEWL, and colorimetry. Petrolatum was found to be an effective barrier cream against SLS, NaOH, and lactic acid and moderately effective against toluene. Frosch et al. (12) revised the RIT (see Section "Animal models") to evaluate the effect of two barrier creams in preventing SLS-induced irritation. The irritant was applied to the ventral forearms of human subjects for 30 minutes daily for two weeks. Visual scoring, LDF, colorimetry, and TEWL were used to assess resultant erythema. TEWL was found most useful in quantifying results, whereas colorimetry was the least beneficial.

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37 Physiologically based pharmacokinetic modeling of dermal absorption

James N. McDougal and James V. Rogers

INTRODUCTION

As the largest organ of the body, the skin fulfils many roles that include barrier function, acting as a physiologic and sensory mediator, and serving as a conduit between the external environment and internal biological processes. The skin physically resists desiccation, infection, chemical penetration, and ultraviolet light damage, while physiologically regulating temperature, providing mechanical and chemoprotective support, and responding immunologically. The skin is an effective biological communicator that influences intricate cellular and molecular networks both locally and systemically, and is an easily accessible organ for evaluating toxicologic endpoints through minimally invasive tissue sampling.

Understanding and quantifying the processes associated with chemical penetration into and through the skin is important in both pharmacology and toxicology. The human species is of primary interest; however, laboratory animals are often used as surrogates, particularly in toxicologic studies. Thus, understanding differences between species is important for extrapolating laboratory-based data to humans in a meaningful way. In vivo studies are advantageous over in vitro studies in which the intact skin has blood flow, is metabolically active, and possesses nervous and humoral responses. Traditional analysis of in vivo skin penetration has involved the estimation of the amount of chemical that has penetrated using either chemical blood concentrations or the amount excreted following dermal exposure. These methods are descriptive due to their secondary indication of chemical concentration that has penetrated into or through the skin. The applicability of these results is limited to the specific experimental design and the similarities between the laboratory species chosen and humans.

For decades, physiologic and pharmacokinetic principles have been characterized mathematically as a feasible alternative for analysis of in vivo skin penetration. These physiologically based pharmacokinetic (PB-PK) approaches describe the dynamics of chemicals in the body in terms of blood flow rates, membrane permeability, and chemical partitioning into tissues. Characterizing cutaneous chemical absorption in terms of biological and physiologic parameters that are measurable and species-specific helps to facilitate extrapolations to humans providing these parameters are known or can be determined. This chapter describes PB-PK models for quantifying and understanding processes of dermal absorption and penetration, and their suitability for dose, route, and species extrapolation.

WHY USE PB-PK MODELS?

An advantage of dermal PB-PK models over traditional in vivo methods is the ability to describe nonlinear biochemical and physical processes. In skin, characterizing chemical penetration in terms of "percent absorbed" assumes that all processes underlying absorption and penetration possess a linear relationship with the exposure concentration. However, skin penetration may not be linear due to differential binding, metabolism, and blood flow. Therefore, factoring in nonlinear processes, such as absorption, distribution, metabolism, or elimination of a chemical, using "percent absorbed" as a description for cutaneous penetration may not be accurate. Many biochemical processes in the body are nonlinear. For example, the percent of chemical metabolized per hour at a low liver concentration may be much greater than the percent metabolized per hour at a high liver concentration. A quantitative description of saturable kinetics in the model may enable the prediction of blood or tissue concentrations from various doses. A complete mathematical description of dermal pharmacokinetics factors in whole body mass balance, and makes it possible to estimate fluxes (amount/time) and permeability constants (distance/time). These expressions of the penetration process are required to accurately predict chemical penetration in various exposure scenarios (e.g., different exposure area, time, or concentration) when nonlinear processes are known to be present.

A properly validated PB-PK description of the skin can provide more information than from experimentation alone. For example, if a chemical concentration in an organ or tissue is an important toxicologic endpoint, understanding the quantitative relationship between blood concentrations and tissue concentrations may provide the tissue dose estimate without the need for invasive tissue sampling. Another example would be the estimation of metabolism rates in the skin where comparing a PB-PK description of known compound metabolite production with the rates of metabolite production following dermal application, the metabolic parameters in the skin could be estimated.

Before any experimentation, PB-PK models can often be used to form predictions that will help in designing experimental doses and sampling times to avoid "range finding" experiments. During the experiment, PB-PK descriptions may allow the use of fewer animals because it may not be necessary to evaluate at many time points to get tissue concentrations. After the study is complete, PB-PK models allow one to extrapolate results to other exposure areas, times, or concentrations, possibly eliminating the need to repeat an experiment under different conditions.

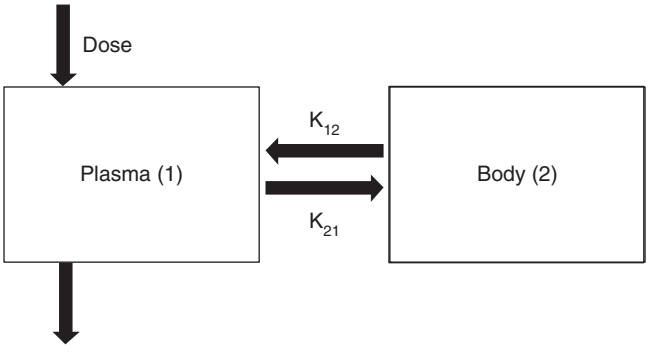

FIGURE 37.1 Classical pharmacokinetic model with two compartments and first-order transfer and elimination rates.

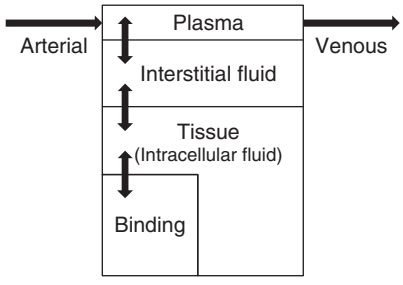

FIGURE 37.2 Diagrammatic description of a lumped compartment with three subcompartments and binding in the tissue subcompartment.

Another important reason for using PB-PK models of skin penetration is the extrapolation to other species. Classical pharmacokinetic modeling assumes that the body can be adequately described by one to three compartments based on the shape of the semilogarithmic plot of plasma concentration versus time (1). The most common classical description is a two-compartment linear system where one compartment is the plasma and the other are all the remaining body water and tissues. Using this type of model, the plasma concentration curve can be fit by a distributive phase and a postdistributive phase. This type of model is useful in clinical situations for determining dose or dose regimen. Classical modeling has occasionally been used in skin penetration studies (2–8).

Figure 37.1 is a schematic representation of the classical two-compartment pharmacokinetic model having a body compartment connected with the plasma in which the first-order transfer rates (K_{12}, K_{21}, K_{10}) are descriptive of a particular situation (1) but do not allow extrapolation to other exposure conditions or species due to physiologic obscurity. PB-PK models are better suited for extrapolation because their physiologic basis is well defined. This was demonstrated by Ramsey and Andersen (9) where a PB-PK model for styrene inhalation in rats could be predictive of blood and exhaled air styrene concentrations in humans after adjusting the physiologic and metabolic constants. Extrapolation with a PB-PK model is only limited by the ability to quantitatively describe the species differences with respect to the pharmacokinetic and physiologic processes involved.

PB-PK MODEL COMPONENTS

In general, a mammalian organism comprises diverse fluid pools (some metabolically active) separated by membranes that obey the physical laws of fluid dynamics, transport, and diffusion, enabling them to be characterized mathematically. The major fluids are blood plasma, interstitial fluids, and intracellular fluids that contribute 60% of body weight. Plasma is the most important fluid due to its continuous motion that transports the red blood cells, white blood cells, platelets, and soluble components in the blood. Interstitial fluid is separated from the plasma only by capillary walls and bathes cells with three times the volume of plasma. The comparatively static intracellular fluid is separated from the extracellular fluids by specialized cell membranes with sophisticated, highly controlled transport systems. The membranes in the tissues that keep these fluids organized are protein–lipid structures of varying thicknesses, which may contain alterable apertures and carry metabolic enzymes. Using these uncomplicated descriptions, most pharmacokinetic processes can be simplified and described in terms of flows, volumes, solubilities, diffusion, and metabolic rates. When these physiologic and biochemical processes can be quantified, a mathematical description can be constructed and compared with empirical data to accurately describe the processes involved (10–13).

Tissue Compartments

The building blocks of a PB-PK model are the compartments. A compartment is a collection of fluids or tissues and/or organs that are grouped together based on physiologic and pharmacokinetic characteristics rather than anatomic properties (13). Tissue compartments can be either lumped together or split. Lumping can be considered as grouping those tissues that are pharmacokinetically and toxicologically "indistinguishable." Splitting compartments is based on the assumption that these tissues are pharmacokinetically and toxicologically "distinct." Each lumped compartment receives inward flux of chemical in the blood flow, possesses a defined volume, and may incorporate binding or loss of chemical through outward flux or metabolism. Subcompartments may be necessary to accurately describe the barriers to movement or sequestration of chemical (Fig. 37.2). The relationship of lumping and splitting within a PB-PK model structure is shown in Figure 37.3.

Such levels of complexity may not always be necessary to adequately describe the physiologic and kinetic processes that are occurring. Chemical transport across the thin compartmental membranes may be so rapid that the plasma and interstitial fluid have equivalent chemical concentrations; therefore, it may be possible to combine the plasma and interstitial fluid into one extracellular fluid subcompartment. Chemical diffusion across cellular membranes into the intracellular fluid may be so rapid that blood flow to the compartment is the rate-limiting factor affecting chemical uptake, thereby possibly avoiding subcompartments completely. However, the free chemical concentration in the plasma, interstitial fluid, or intracellular fluid subcompartments will depend on whether binding or metabolism occurs in each subcompartment.

General PB-PK Model

Chemical penetration through the skin is a process that lends itself to PB-PK modeling. Compartments are chosen based on an understanding of the pharmacokinetics of the chemical and the purpose for the model. Figure 37.4 shows a model with five simple compartments that was designed for predicting blood concentrations from different cutaneous exposure times and concentrations. Each

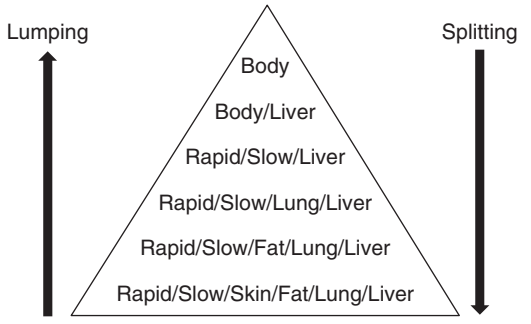

FIGURE 37.3 Diagrammatic representation of compartment lumping and splitting.

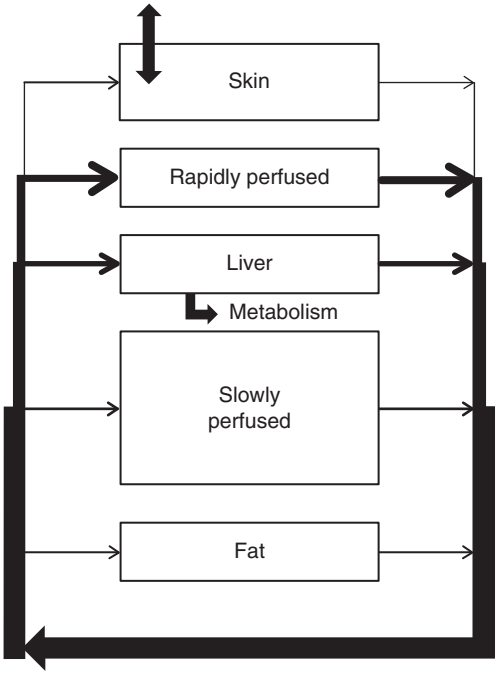

FIGURE 37.4 Diagrammatic representation of a PB-PK model with five simple compartments connected by blood flow. Compartment volumes and blood flows are approximately to scale.

compartment is assumed to be well-stirred, flow limited, and has no subcompartments. Potential chemical loss could be from evaporation, hepatic metabolism, and exhalation. The description is of the venous equilibration type, without blood volume being specified.

The rapidly perfused compartment lumps tissues with high blood flow, and high chemical affinity, such as kidney, viscera, brain, and other richly perfused organs. The slowly perfused compartment has low blood flow, low chemical affinity, and represents muscle and other poorly perfused tissues and organs. The fat compartment has low blood flow, high affinity for the chemical, and represents various types of fat. According to this description, the sole route of entry for the chemical is the skin and elimination is by way of diffusion out of the skin followed by metabolism in the liver, and exhalation (if the chemical is volatile). Additional compartments would be required for a chemical eliminated in the kidney, or concentrated in a target organ.

Skin Compartment

The skin compartment is a special subset of tissues with defined anatomic and physiologic properties. Figure 37.5 illustrates a skin

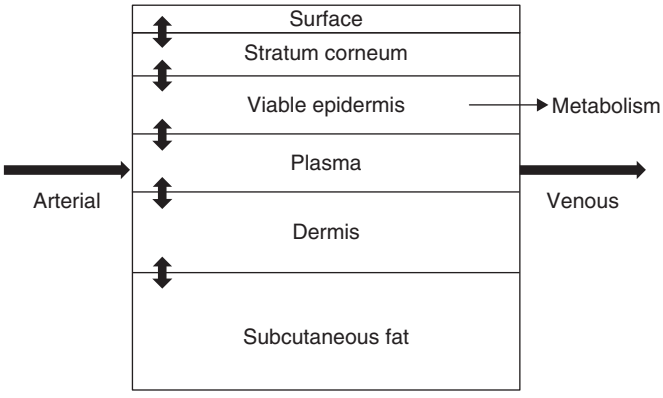

FIGURE 37.5. Diagrammatic representation of a skin compartment with six subcompartments and metabolism occurring in the viable epidermis.

compartment that contains most of the anatomic detail that may be important in skin penetration (14,15). Most of this detail will not be necessary for all chemicals, but is described here for completeness. Each subcompartment communicates bidirectionally with adjacent compartments and possesses a concentration, volume, and affinity for the chemical of interest. The surface subcompartment is crucial to making the PB-PK model functional as the volume, surface area exposed, concentration, amount applied to skin, and affinity of the chemical for the vehicle (if any) are all incorporated into this subcompartment. If evaporation occurs or if the chemical is applied in a vehicle that has a characteristic penetration rate, the event must be incorporated into the description to account for the penetration driving force.

The stratum corneum subcompartment is the principal barrier to penetration for most chemicals due to the compactness of its lipid–protein matrix (16–20). Although the stratum corneum can act as a reservoir for lipophilic chemicals and may provide binding sites, there is little (if any) metabolic activity or any active transport processes (20). The stratum corneum subcompartment is treated as if it were homogenous and well stirred; however, this gross oversimplification will not apply for all chemicals. For some chemicals, it may be necessary to model the stratum corneum as a multilayered structure (21,22). The stratum corneum permeability has been mathematically described in terms of bilayer-scale transport and the characteristic dimensions of the stratum corneum microstructure (23). These stratum corneum permeability expressions, specifically effective tortuosity, have been incorporated into a PB-PK model of organophosphate dermal absorption (24).

The viable epidermis subcompartment contains cells formed in the basal layer that differentiate and become keratinized and more compact as they migrate toward the surface to form the stratum corneum. The majority of the metabolic activity of the skin is found in this layer and it may provide chemical binding sites (25–27).

The plasma subcompartment in the skin provides blood flow to the dermis. Its vasculature is neurally regulated, provides nutrients and other essential components to the skin, and provides body heat dissipation from the extremities. Pharmacokinetically, the plasma subcompartment receives chemicals penetrating the skin and from arterial blood. Chemicals leave the skin via the venous blood or by metabolism. Although embedded in the papillary dermis, the plasma subcompartment in this simplified description is between the viable epidermis and the dermis (22,28).

The dermis subcompartment provides structural support for the epidermis. It consists of a thick fibrous matrix of elastin and collagen

and is more porous than the other compartments. The collagen in the dermis constitutes approximately 77% of the dry mass of the skin (22). Chemicals may bind to these structural components as they transit through the skin. The upper part of the dermis contains capillaries that provide nutrients to the viable epidermis. The subcutaneous fat subcompartment represents a layer of variable thickness, which is poorly perfused but may provide a reservoir for lipophilic chemicals. Although below the level of the capillary beds, its perfusion suggests that it could be an important compartment in its own right.

Although the subcompartments make the skin compartment complex for modeling purposes, it is still an oversimplification of the actual intricacy of mammalian skin. Notably missing are appendages (sweat glands, hair follicles, and sebaceous glands), which have been suggested to be contributing pathways for absorption at early times with slowly diffusing electrically charged chemicals (19,20). Bookout and collaborators (15) have described physiologically based modeling of appendages.

Flux Equations

Flux equations are the key to an appropriate model (29). The rate of change of amount (product of volume and concentration) in a subcompartment at any time is a balance between inward and outward flux:

$$V\frac{dC}{dt} = Influx_{\text{total}} - Efflux_{\text{total}} \tag{37.1}$$

where V is the volume, C is the free concentration (mass/volume), and *Influx* and *Efflux* are sums of the fluxes (mass/time) in each direction [Eqs. 37.2–37.4 below]. The general form for the equation describing unidirectional flux where transportation of a chemical is occurring because of bulk flow of the medium is:

$$Flux = QC \tag{37.2}$$

where Q is flow (volume/time) of the medium.

When membranes between subcompartments (e.g., capillary or cell membrane) act as barriers to simple diffusion or when adjacent compartments (e.g., viable epidermis and dermis in Figure 37.5) act as though there is a membrane between them, the flux from outside to inside is described by the permeability–area product and the concentration difference across the membrane:

$$Flux = PA(C_{\text{out}} - C_{\text{in}}) \tag{37.3}$$

where P is permeability (distance/time), A is area (distance^2), and C_{out} and C_{in} are the free concentrations at the outer and inner surfaces of the membrane. The thermodynamic activity differential drives the transport process. Therefore, if the chemicals across the barrier are in different media, it is the effective concentration at the interface that must be used in the calculation and the concentration must be adjusted for partitioning between the different media.

In some cases, movement across a barrier between subcompartments may not be by simple diffusion. If there is a saturable, active process involved, the description for flux is often represented by:

$$Flux = \frac{kVC}{K + C} \tag{37.4}$$

where k is the maximum transport rate (mass/volume × time), and K_T is the Michaelis-like constant (mass/volume).

Binding, Metabolism, and Excretion

The free chemical concentration in a subcompartment can also be reduced by binding to proteins or cellular macromolecules, metabolic processes, and excretion (11,13). Normally, these processes are either first-order, saturable, or some combination of the two. If the process is first-order, the general equation is:

$$Loss = rCV \tag{37.5}$$

where *Loss* has the same units as *Flux* (mass/time) and r is a proportionality constant (time^{-1}). This description of loss will have the same form regardless of whether the first-order loss is due to irreversible binding, metabolism, or excretion. When the binding, metabolism, or excretion is saturable, the loss can be described by an equation of the same form as Equation (37.4) (11,13). The equation for saturable metabolism is:

$$Loss = \frac{V_{\max}C}{K_{\text{m}} + C} \tag{37.6}$$

where $V_{\max}$ is the maximum reaction velocity (mass/time) and K_{m} is the Michaelis constant.

Mass Balance Equations

Each Lumped Compartment

For each subcompartment in Figure 37.2, a differential equation in the form of Equation (37.1) can be constructed. Equations (37.7–37.9) (9) are for plasma, interstitial fluid, and intercellular fluid in tissues, respectively:

$$V_{\text{p}}\frac{dC_{\text{p}}}{dt} = Q_{\text{t}}(C_a - C_v) + P_{\text{is}}A_{\text{is}}\left(\frac{C_{\text{is}}}{R_{\text{is/p}}} - C_{\text{p}}\right) \tag{37.7}$$

$$V_{\text{is}}\frac{dC_{\text{is}}}{dt} = P_{\text{is}}A_{\text{is}}\left(C_{\text{p}} - \frac{C_{\text{is}}}{R_{\text{is/p}}}\right) + P_{\text{t}}A_{\text{t}}\left(\frac{C_{\text{t}}}{R_{\text{t/is}}} - C_{\text{is}}\right) \tag{37.8}$$

$$V_{\text{t}}\frac{dC_{\text{t}}}{dt} = P_{\text{t}}A_{\text{t}}\left(C_{\text{is}} - \frac{C_{\text{t}}}{R_{\text{t/is}}}\right) - rC_{\text{t}}V_{\text{t}} \tag{37.9}$$

where subscripts p, is, and t refer to the plasma, interstitial, and tissue (intercellular fluid) subcompartments, respectively. C_{a} is concentration in the arterial blood, C_{v} is the concentration in venous blood, and R is the partition coefficient between the media indicated by its subscripts. The concentration in the lumped compartment is the volume average of the concentration of the subcompartments:

$$C_{\text{i}} = \frac{C_{\text{p}}V_{\text{p}} + C_{\text{is}}V_{\text{is}} + C_{\text{t}}V_{\text{t}}}{V_{\text{p}} + V_{\text{is}} + V_{\text{t}}} \tag{37.10}$$

Each of the compartments in the model shown in Figure 37.4 could require treatment as a diffusion-limited lumped compartment; however, the simplification shown in Equation (37.18) adequately describes the pharmacokinetic behavior of many lipid-soluble organic chemicals.

Skin Compartment

For skin subcompartments in Figure 37.5, Equations (37.11–37.17) account for mass fluxes within each subcompartment:

$$V_{sfc}\frac{dC_{sfc}}{dt} = P_{sc}A_{sc}\left(\frac{C_{sc}}{R_{sc/sfc}} - C_{sfc}\right) \tag{37.11}$$

$$V_{sc}\frac{dC_{sc}}{dt} = P_{sc}A_{sc}\left(C_{sfc} - \frac{C_{sc}}{R_{sc/sfc}}\right) + P_{ve}A_{ve}\left(\frac{C_{ve}}{R_{ve/sc}} - C_{sc}\right) \tag{37.12}$$

$$V_{ve}\frac{dC_{ve}}{dt} = P_{ve}A_{ve}\left(C_{sc} - \frac{C_{ve}}{R_{ve/sc}}\right) + P_{d}A_{d}\left(\frac{C_{d}}{R_{d/ve}} - C_{ve}\right) - \frac{V_{max}C_{ve}}{K + C_{ve}} \tag{37.13}$$

$$V_{d}\frac{dC_{d}}{dt} = P_{d}A_{d}\left(C_{ve} - \frac{C_{d}}{R_{d/ve}}\right) + P_{p}A_{p}\left(\frac{C_{p}}{R_{p/d}} - C_{d}\right) \tag{37.14}$$

$$V_{p}\frac{dC_{p}}{dt} = Q_{sk}(C_{a} - C_{v}) + P_{p}A_{p}\left(C_{d} - \frac{C_{p}}{R_{p/d}}\right) + P_{sf}A_{sf}\left(\frac{C_{sf}}{R_{sf/p}} - C_{p}\right) \tag{37.15}$$

$$V_{sf}\frac{dC_{sf}}{dt} = P_{sf}A_{sf}\left(C_{p} - \frac{C_{sf}}{R_{sf/p}}\right) \tag{37.16}$$

where the subscripts sfc, sc, ve, d, p, sf, and sk stand for surface, stratum corneum, viable epidermis, dermis, plasma, subcutaneous fat, and skin, respectively. The concentration in the skin as a whole is the volume average of the concentration of the subcompartments:

$$C_{sk}\frac{C_{sc}V_{sc} + C_{p}V_{p} + C_{ve}V_{ve} + C_{d}V_{d} + C_{sf}V_{sf}}{V_{sc} + V_{p} + V_{ve} + V_{d} + V_{sf}} \tag{37.17}$$

It must be emphasized that these are theoretic descriptions of skin penetration. These compartments have been chosen based on the current understanding of what may be the most important structural components involved. Exploration and understanding of these concepts will determine those that are important subcompartments for each specific chemical to be studied.

Simplifying Assumptions

For completeness, the hypothetical compartments in Figures 37.4 and 37.5 have been described using the PB-PK approach to diffusion limitation in each subcompartment. However, until methods are developed to measure the permeability–area products (*PA*) for subcompartment interfaces, many simplifications must be made to make the description useful for extrapolation. One simplifying approach has been to lump *P* and *A* together into a single term that has units of volume/time and is estimated or fit (13,30–32). A problem with combining *P* and *A* is the lack of knowledge about scaling from one species to another. It has been assumed that the permeability term is related to a constant physical process across species and the area can be scaled according to body weight (31). Moreover, considerations must be made for differences in permeability rates that vary among different regions of the body (33).

There are several assumptions that have been used to collapse the subcompartments shown in Figures 37.2, 37.4, and 37.5 that reduces the complexity of the problem. When transfer across the cell membrane is the rate-limiting step, the plasma and interstitial subcompartments can be combined into a single extracellular compartment (11,13). When blood flow to the tissue is the rate-limiting step, all subcompartments can be collapsed into a single well-stirred compartment where the rate of change in compartment chemical concentration as a whole is related to blood flow and the difference between arterial and venous blood concentrations (34–41), which is a consolidation of Equations 37.1 and 37.2:

$$V_{i}\frac{dC_{i}}{dt} = Q_{i}\left(C_{a} - \frac{C_{i}}{R_{i/b}}\right) \tag{37.18}$$

where the *i* subscript refers to any compartment and $R_{i/b}$ is the partition coefficient between the tissue and the blood. It has also been assumed that the concentration of chemical in tissue is in equilibrium with mixed venous blood. The tissue concentration (C_i) divided by the tissue to blood partition coefficient is substituted for the concentration in venous blood, assuming the equilibrium condition:

$$R_{i/b} = \frac{C_{i}}{C_{v}} \tag{37.19}$$

where C_v is the chemical concentration in venous blood leaving the tissue.

Full PB-PK Model

When differential equations are written for the skin and body compartments, they need to be connected such that total mass is conserved. The mass balance in the liver compartment is the same as Equation (37.18) except for the addition of saturable metabolism [Eq. (37.6)]:

$$V_{l}\frac{dC_{l}}{dt} = Q_{l}\left(C_{a} - \frac{C_{l}}{R_{l/b}}\right) - \frac{V_{max}\dfrac{C_{l}}{R_{l/b}}}{K + \dfrac{C_{l}}{R_{l/b}}} \tag{37.20}$$

where C_l is the concentration in the liver. The simple skin compartment in Figure 37.4 can be described as a single well-stirred compartment with simple diffusion:

$$V_{sk}\frac{dC_{sk}}{dt} = Q_{sk}\left(C_{a} - \frac{C_{sk}}{R_{sk/b}}\right) + P_{sk}A_{sk}\left(C_{sfc} - \frac{C_{sk}}{R_{sk/sfc}}\right) \tag{37.21}$$

The first term on the right side of the equation describes the effect of blood flow, and the second term is the net flux of chemical into the skin from the skin surface.

The chemical concentration in mixed venous blood is the flow-weighted average of all the concentrations leaving a compartment:

$$C_{v} = \frac{\sum_{i}(Q_{i}C_{i})}{Q_{c}} \tag{37.22}$$

where Q_c is cardiac output (total blood flow).

Model Parameters

The parameters required for the model depend on the compartments chosen based on pharmacokinetics. It is important to know which parameters are available, or can be determined, because

they may be the limiting factors in the structure of the model. Physiologic parameters for rats using a model for volatile lipophilic chemicals have included compartment as a function of blood flow as percentage cardiac output and volume as a percentage of body weight (42). It is important that the sum of the individual blood flows equals the total cardiac output. The sum of the volumes of the compartments only accounts for 91% of the body weight. The other 9% that is not accounted for is nonperfused tissue, such as fur, crystalline bone, cartilage, and teeth.

Chemical-specific parameters of a model are partition coefficients, binding coefficients, and metabolic rates. Partition coefficients are essential components of physiologically based models and describe the ratio of chemical concentrations in different materials at equilibrium and reflect chemical solubility in biological fluids and tissues. Some of the partition coefficients determined by Gargas and coworkers (43) have been used for a PB-PK model of dermal absorption of organic vapors (44). These partition coefficients for volatile chemicals were measured by determining, at equilibrium, the ratio of concentrations in the blood or tissue to the concentration in air. Tissue/blood partition coefficients can be estimated by dividing the tissue/air partition coefficient by the blood/air partition coefficient. Jepson and coworkers (45) developed a method to measure blood/saline and tissue/saline partition coefficients for nonvolatile chemicals (<1 mmHg at 20 C) using filtration under pressure. In this case, tissue/blood partition coefficients can be estimated by dividing the tissue/saline partition coefficient by the blood/saline partition coefficient.

Knowing the chemical concentration in the target tissue or cells during chemical exposures is necessary to characterize the relationship between the chemical concentration and observed biological responses. In skin, a crucial step in the development of a predictive model is to be able to calculate the chemical concentration at the target site (e.g., epidermis) as a function of exposure concentration and surface area exposed. A potential way to address this is to use partition coefficients determined for cellular and extracellular components within a tissue. Rogers and McDougal (46) demonstrated an approach for calculating partition coefficients of volatile chemicals for cells in which cytotoxicity could be calculated in terms of cellular chemical concentration. This was accomplished by first calculating the cell culture medium:air ($PC_{m/a}$) and fibroblast:air ($PC_{f/a}$) partition coefficients for m-xylene using the method of Gargas and coworkers (43). The formula $C_c = (PC_{f/m})(C_m)$ was used to calculate the cellular chemical concentration where C_c is the m-xylene concentration in the fibroblasts (mass m-xylene/g cells), $PC_{f/m}$ is the fibroblast:medium partition coefficient ratio for m-xylene, and C_m is the chemical concentration in the exposure medium as determined by GC analysis.

Similar in vitro studies showed the use of partition coefficients for volatile chemicals in cells could be used to relate various toxicologic endpoints to cellular chemical concentration (47,48). These studies show that target cell/tissue concentrations can be estimated using partition coefficients and factoring these values into PB-PK models could aid in predicting skin chemical concentrations and resulting toxicity.

Metabolic constants describe the rate of loss of chemical from a lumped compartment. Saturable (V_{max} and K_m) and first-order (K_{fo}) metabolic constants for several volatile organics have been incorporated in a model for describing dermal absorption of chemical vapors (44). Most of these metabolic constants for rats were determined in vivo by gas uptake techniques (49), but they can also be determined in vitro (50–52).

DEVELOPING PB-PK MODELS

PB-PK models are different from multipurpose software programs as the structure of a PB-PK model is dependent on the interaction of a specific chemical with a specific species. Each unique chemical–species interaction requires that the salient physiologic and pharmacokinetic principles be understood and quantitatively described. Development of a PB-PK model is an iterative process that requires insight, trial and error, and careful laboratory investigation. PB-PK models can and should be developed before the first laboratory experiment. As knowledge is gained in the laboratory, each new understanding should be quantitatively described in the model. Simulation and experimentation should be accomplished concurrently. Simulation prior to experimentation will allow appropriate data to be collected. Experimentation will confirm or increase the understanding that is quantified in the model.

Choose Compartments

Decisions about the form of the skin compartment are related to the behavior of the chemical in the skin. Lag time (the time before steady state penetration rate is achieved) is the single most important determining factor. If lag time prior to achieving steady state absorption is short (e.g., 15 minutes), a simple well-stirred homogenous skin compartment (Fig. 37.4) may be an adequate description. If the lag time is longer, it will be necessary to include part or all of the skin subcompartments (Fig. 37.4). Chemical distribution in the skin will determine which compartments are important to describe explicitly. Many of the methods that have been developed to study the skin will be useful for increasing the understanding required for an appropriate mathematical description. These include methods for metabolism and penetration, laser Doppler velocimetry, tape stripping, toxicogenomics/proteomics, and ultrastructural analysis.

In addition to a skin compartment, determining which other compartments to include in a model requires knowledge of the pharmacokinetics of the chemical of interest. Depending on the chemical, pharmacokinetic information may be derived from the literature or it may need to be determined in the laboratory. Compartments must be included in a model to represent the major organs of metabolism and excretion. Metabolism studies with radiolabeled chemicals or other analytical methods will provide the kinetic data required to choose the important compartments. Additional lumped compartments must be included to account for chemical distribution, such as a lipophilic chemical requiring a fat compartment or compartments. Distribution studies with radiolabeled or nonlabeled chemicals provide the details necessary for appropriate choices of compartments. New analytic methods, such as positron emission tomography or nuclear magnetic residence imaging appear promising and may provide valuable distribution information in the whole animal. Organs with similar chemical distribution may be lumped together if the organs have similar blood flow per weight of tissue. Other compartments that may be desired in the model may represent target organs for toxicity, or therapeutic effect.

Decisions about the form of a lumped compartment and the requirement for subcompartments depend on the relationship between blood flow to the compartment, volume of the compartment, and chemical solubility in the compartment. Deciding whether the limiting factor in transfer of chemical from blood to the compartment is flow or diffusion is not always simple experimentally (53). It is probably best to assume blood flow is the limiting

factor unless there is evidence otherwise or flow-limitation does not adequately describe the behavior of a compartment. The most important principle in PB-PK modeling is to use the simplest description that adequately describes chemical behavior.

Determine Physiological Parameters

Species-specific physiologic parameters, such as blood flow and organ volumes, are often available in physiology handbooks, literature reviews, or published PB-PK models. It is necessary to make decisions about which physiologic parameters to use from the literature as there will most likely be a range of values. One must avoid changing the physiologic parameters to fall outside these normal ranges to obtain agreement between prediction and observation. If changes are made then the result could lead to the inability to extrapolate across species and to humans. The physiologic parameters in a PB-PK model for any species should be robust and not change when a different chemical is modeled, unless there is sound evidence that the chemical specifically causes changes. When prediction and observation do not agree there are two explanations: (*i*) either the results of the experiment are not accurate, or (*ii*) the model assumptions are inadequate. Once experimental calculations have been checked, the best approach is to determine if the model structure is adequate.

Determine Chemical Parameters

Metabolic constants, partition coefficients, and binding coefficients are much less available in the literature than the physiologic parameters and must often be determined experimentally. Metabolic constants can be determined both in vitro and in vivo. Methods used for measuring metabolism of volatile chemicals for PB-PK modeling are the tissue homogenate method (50) and in vivo gas uptake method (49). Partition coefficients for volatile chemicals in blood and tissue homogenates can be determined by the vial equilibration technique (43). Partition coefficients for nonvolatile chemicals can be determined by measuring tissue and blood concentrations after continuous dosing to achieve equilibrium. Binding is distinguished from partitioning due to its nonlinearity in relation to concentration and can be determined by various methods (54,55). The same caveat about changing physiologic parameters to fit the data applies to the chemical parameters. Halving the blood/air partition coefficient because it fits the experimental results better may solve an immediate problem at the expense of the general applicability of the model.

Validate Model Where Absorption is Absent

Before a PB-PK model can be used to describe the process of chemical absorption through the skin, it is necessary to gain some confidence in the quantitative description of pharmacokinetics when absorption is absent. The model should successfully simulate blood concentrations, tissue concentrations, or expired breath after intravenous exposures at several concentrations. Urinary or fecal excretion could also be used for validation, but they are not optimum because sampling times are critical. Ideally, prolonged intravenous infusions at several concentrations and intravenous boluses at several concentrations should be used to demonstrate that the physiologic and pharmacokinetic parameters chosen will adequately describe the processes of distribution, metabolism, and excretion for a wide range of concentrations. An alternative approach would be to achieve the same confidence with subcutaneous infusions using minipumps.

Validate Model with Dermal Absorption

Once the parameters not involved in absorption are fixed, then the model can be used to understand the process of absorption through the skin. Parent chemical distribution in the body after absorption through the skin and hepatic metabolism will follow the same principles independent of the absorption process. When these processes are understood and quantified, the rate of absorption through the skin can be determined based on blood, tissue, breath, or excreta concentrations. Permeability constants can be determined by using the model to determine total chemical absorbed as long as the concentration on the skin and the surface area are known (42). Predicted blood concentrations for several organic chemicals in rats dermally exposed to controlled vapor concentrations during whole body exposures with respiratory protection have been demonstrated (42).

Extrapolation to Humans

The ability to extrapolate from laboratory species to man is one of the most important reasons for using PB-PK models. Ramsey and Andersen (9) have shown that a PB-PK model for inhalation of styrene vapors in rodents can predict the pharmacokinetic behavior of inhaled styrene in humans by changing the blood flows, organ volumes, and partition coefficients to those of humans. The same principles could be used to extrapolate dermal absorption studies to humans if differences in skin structure are taken into account. Ultimately, it could be possible to quantify the species differences in blood flow, differences in stratum corneum, epidermal and subcutaneous fat thickness and composition, as well as the effect of the type and number of appendages on skin penetration in various species.

Organic vapor penetration rates determined in rats using a PB-PK model are two to four times greater than penetration rates in humans calculated from the literature based on the total absorbed after whole body exposures (44). The consistency of these comparisons suggests that differences in permeability may be due to physical differences in the skin. Using this as an example, it is important to understand some of the approaches and limitations involved in extrapolation to humans. It is not possible to directly extrapolate, with any confidence, the published PB-PK model for organic vapors in rats to the published human studies. This is because the human studies were based on urinary output and/or exhaled breath and the rat studies were based on blood concentrations. The rat model could be more predictive of exhaled breath and urinary output by adding urinary output and validating the rat model for these routes of excretion.

Once the rat model accurately predicted experimental results for urinary output and exhaled breath, the rat model could be used to address the human data by changing the physiologic, pharmacokinetic, and biochemical parameters in the model to those of the human. For example, alveolar ventilation rates, blood flows, organ volumes, and urine volumes would need to be changed to those of the human. Partition coefficients, metabolic rates, and urinary excretion rates would need to be found or determined for each chemical of interest and changed in the model. With the published rat description, permeability constants were determined with confidence because the model was validated with a route where complex

absorption via inhalation was absent. If the scaled-up rat model did not predict the dermal exposures in humans, it could be attributed to a different permeability constant in humans (as suspected) or because the physiologic or pharmacokinetic parameters used for humans were incorrect. It would be necessary to make sure that these parameters were correct in humans with a route of absorption other than dermal.

Providing the rest of the description was correct, any inaccuracy in the prediction would be due to differences in permeability constant in the skin, and the permeability constant could be estimated by determining the constant required to fit the data. If the simple skin compartment [Eq. (37.21)] were descriptive for this chemical in the rat, it would most likely be descriptive of the same chemical in the human. Other types of chemicals, which penetrate more slowly than organic vapors, may require that the skin be broken into some or all of the subcompartments described in Equations (37.11–37.16). In such a case, the subcompartments would also require that the structural differences in the skin between species be understood and quantified.

Other types of skin models using first-order rate constants to describe chemical transfer between subcompartments are excellent descriptive models. However, these models do not extrapolate to other species well, because the first-order rate constant is a composite of the permeability, area exposed, and the partition coefficient. These models have been reviewed and compared with other PB-PK models (56,57).

When the Model Fails

Models are often most useful when they fail to adequately describe the experimental data. During the process of developing a more adequate description of the pharmacokinetic processes, further insight can be gained to increase the understanding of chemical pharmacokinetics in the skin. The physiologic properties of the living system are the foundation of the model description that forces an investigator to design experiments to elucidate where the model description is inaccurate. Chemicals and biological systems interact in accordance with physicochemical principles and PB-PK modeling of these principles is an iterative process that requires theory and observation to amalgamate until the final result is achieved.

Value of PB-PK Skin Models

PB-PK modeling can increase the understanding of the effect of vehicles on penetration rates and penetration enhancement. Jepson and McDougal (58) showed the importance of the skin/vehicle partition coefficient by demonstrating that permeability measured in vivo could be extrapolated between different vehicles (water, corn oil, and mineral oil) with a reasonable degree of accuracy. Traditionally, flux measurements must be made on a system that is at or near steady state. Jepson and McDougal (59) demonstrated that a PB-PK model could be used as a tool to estimate in vivo permeability in a situation where steady state is never achieved, such as an organic chemical in a small volume of water. These models have also accurately described in vitro skin and receptor solution concentrations in the first 20 minutes of organic chemical in aqueous vehicle (60). Real-time breath analysis has been linked with PB-PK modeling as a tool to investigate human dermal absorption of volatile chemicals from water (61,62) and soil (62,63). These noninvasive approaches would not have been possible without a PB-PK model to estimate body burden.

Future of PB-PK Skin Models and Final Thoughts

Improved skin compartments can be developed and validated that include some of the subcompartments shown in Figure 37.5 to be predictive of chemical penetration rates that have more complicated absorption profiles. Pharmacodynamic models that quantitatively describe the molecular events that occur in the skin with local toxicity (e.g., psoriasis, contact dermatitis, or skin cancer) can be developed. These models might describe chemical concentrations in tissues, production and turnover rates of important proteins, signaling molecule activity, differential mRNA expression, RNA interference, biomarkers, and other molecular circuitry patterns that are responsible for changes in skin physiology. Identifying highly specific molecular/physiologic indicators and improved mathematical modeling approaches is a critical component for improving human health in the future. Such approaches could be used to develop a more "personalized" application for individuals or populations at risk for disease or genotoxicity. For example, Ierapetritou and coworkers (64) describe the use of in vitro and in silico liver models for generating a "virtual organ" system and its potential application to clinical evaluations of drug efficacy and toxicity. With appropriate biologically based models, it is possible to understand the relationship between the amount of chemical on the skin surface and the resultant therapeutic or toxic effect.

PB-PK models provide an increased capacity in the understanding of skin absorption and effects of chemicals both locally and systemically. The ability to extrapolate between in vivo exposure conditions, doses, and species allows for laboratory experimentation to provide an abundance of information applicable to human exposure situations that can be applied to exposure and risk assessment. The application of quantitative descriptions to processes occurring in the skin is limited only by the ability to fully characterize and understand the physiologic and biochemical processes involved. Model validation could ultimately lead to the development of a specific panel of biomarkers and targeted therapeutic or prophylactic treatment.

NOMENCLATURE

C	Concentration (mass/volume)
V	Volume
A	Area (distance^2)
Q	Flow (volume/time)
P	Permeability (distance/time)
K_m	Michaelis metabolic constant (mass/volume)
K_T	Michaelis-like transport constant (mass/volume)
k	Maximum transport rate (mass/volume × time)
r	Proportionality constant (time^{-1})
R	Partition coefficient (unitless, ratio of concentrations)
V_{max}	Maximum velocity (mass/time)

Subscripts

a	arterial
b	blood
c	cardiac output
d	dermis
e	extracellular
i	ith tissue
is	interstitial
p	plasma

sc	stratum corneum
sf	subcutaneous fat
sfc	surface
sk	skin
t	tissue
v	venous
ve	viable epidermis

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38 In vitro approaches to the assessment of skin irritation and phototoxicity of topically applied materials

Chantra Eskes, Joao Barroso, and David Basketter

INTRODUCTION

Irritation, with or without the involvement of solar radiation, is a relatively common phenomenon. Skin irritation arising directly as a consequence of exposure (deliberate or accidental) to materials is typically experienced as cumulative irritant contact dermatitis. Acute irritation arising from a single contact is less common, and largely comprises of corrosive effects from very aggressive materials. In this chapter, the most advanced *in vitro* methods for the identification of corrosivity, irritancy, and phototoxicity are reviewed, their validation status documented, and their utility in the completion of safety assessment (regulatory and for occupational/consumer exposure) is considered. Finally, challenges for the future are detailed, particularly in the context of the relevance of existing validated *in vitro* procedures and their use in the regulatory and industrial framework, for establishing safety use exposures for topically applied materials.

SKIN IRRITATION

Skin irritation is a deceptively simple phenomenon. The reality, however, is very different: Skin irritation responses range from a variety of sensory effects, through minor degrees of acute reaction, characterized by erythema, or cumulative irritancy, characterized by erythema and dryness, to more profound degrees of response, including burning/corrosion, with consequent scar formation. Such matters are fully covered elsewhere (e.g., Chew and Maibach, (1), as well as in this book), but for the purpose of this chapter, the focus must be on the evaluation of the acute irritant response. This is simply a reflection of the historic use of the rabbit Draize test (2) as a means of identifying those substances that present a skin irritation hazard (3), EU (4), EU, 2008). In this test, a four-hour semi-occlusive treatment with an undiluted material, followed by assessments of erythema and edema during the first 72 hours and up to 14 days, in three rabbits, has been deployed, to discriminate between substances in the following categories:

- Causes severe burns
- Causes burns
- Irritant
- Mild irritant
- Not classified

These categories are rather arbitrary; "not classified" means simply not irritant enough to present an acute hazard. In many areas, including in the European Union (EU), there is no separation between the irritant and mild irritant category. The relevance of these classifications to real human hazard has been questioned for many years (5,7,8). They have almost no place at all in the assessment of the risk to human health, except for materials that can cause burns (9) where the obvious advice is to avoid any direct skin contact with the neat material. However, for irritant materials, the cumulative effects of the formulation, rather than the response to a single exposure of an isolated chemical, is overwhelmingly the issue (12,13).

Set against the above background, there has been considerable progress on the use of *in vitro* models to predict skin irritation (14–16). Moreover, several *in vitro* methods for skin corrosion and irritation have been validated and regularly adopted by the Organization for Economic Cooperation and Development (OECD) and the EU, for over the last 15 years (see Table 38.1 for an overview). In the countries adopting the EU legislation, these assays currently allow the full replacement of animal testing for identifying and classifying compounds such as skin corrosives, skin irritants, and non-irritants. However, a number of questions still remain open regarding their regulatory and industrial applicability. As an example there is an important division between *in vitro* approaches and the identification of corrosive materials and methods, which aim to separate irritant substances from those with little effect on the skin. The identification of corrosive substances has proven to be a relatively simple task, with an optimal strategy falling out readily from the handful of methods officially regarded as validated, which have been proposed to address this endpoint (see chapter below). This contrasts with the position for the differentiation of irritant/mild irritant/non-irritant discrimination, despite the recent adoption of valid scientific methods. It is not appropriate to undertake a detailed/historical review here; instead, the main points are highlighted and illustrated below.

In the past, skin irritation was assumed to be a fairly simple response to direct toxic insult, and thus likely to be readily predicted by observations of the response of cells in vitro to chemical exposure, particularly by measurements of cytotoxicity, for example, the release of the cytoplasmic enzyme lactate dehydrogenase (17) or the release of mediators such as arachidonic acid (18). Although the cells deployed for this purpose were varied, historically, dermal fibroblasts were easy to culture, but keratinocytes

TABLE 38.1
Validated and Adopted In Vitro Methods for Skin Corrosion and Skin Irritation and their Status of Validation and Regulatory Acceptance

Purpose	Test Method	Status
Skin corrosion	Reconstructed human Epidermis models – EPISKIN™ – EpiDerm™ – SkinEthic™ – EST-1000	Validated and adopted (OECD TG 431)
	Transcutaneous Electrical resistance (TER) test	Validated and adopted (OECD TG 430)
	Membrane barrier test – Corrositex®	Validated and adopted (OECD TG 435)
Skin irritation	Reconstructed human Epidermis (RhE) models – EPISKIN™ Skin Irritation Test (SIT) – EpiDerm™ EPI-300-SIT – SkinEthic™ SIT[42 bis]	Validated and adopted (OECD TG 439)

were usually favored as the main cells of the epidermis (19–21). In more recent years, the focus has moved very largely to the deployment of three-dimensional (3-D) skin models, largely based on the argument that these provide a skin barrier (of sorts), and will thus help to take into account the excessive bioavailability of materials applied directly to a monolayer culture, this being one reason proffered for the failure of simple keratinocyte systems to predict a skin irritation hazard with any real accuracy (22–25).

VALIDATION STATUS OF IN VITRO ASSAYS FOR CORROSION AND IRRITATION

Corrosion

Three methods for assessing skin corrosion have been validated and accepted into international regulations. These are the rat skin transcutaneous electrical resistance (TER) test, the human skin model tests (such as EpiSkin™, EpiDerm™, SkinEthic™, and CellSystems®EST-1000), and the membrane barrier test (Corrositex®). Prevalidation of these assays was carried out under the auspices of the European Center for the Validation of Alternative Methods (ECVAM), where the rat skin TER, a human skin model (Skin^{2}™), and Corrositex®, were tested in a blind trial, using 25 corrosives and 25 non-corrosives, by two or three laboratories (26). The recommendations made from this prevalidation led to the carrying out of a formal validation study of these methods (27,28). In the validation trial 60 coded chemicals were tested by three laboratories in each assay, and in addition to Skin^{2}™, EpiSkin™ was included as a second human skin model. During the trial TER, EpiSkin™, and Corrositex® proved to be scientifically valid to discriminate corrosives from non-corrosive substances (28–31). However, the commercial production of the Skin^{2}™ skin model ceased, so, further catch-up validation studies were subsequently carried out using other similar, commercially available human skin models. These models, which were subsequently endorsed as also being scientifically valid for skin corrosion testing were: EpiDerm™ (32,31); SkinEthic™ (34,35), and CellSystems®EST-1000 (36,38).Official test guidelines on the TER and on the human skin models were adopted by the EU in 2000 (within Annex V of the Dangerous Substances Directive and later taken up by the EU Test Method Regulation in 2008; EU, (39,40) and by the OECD in 2004 (41,42), whereas Corrositex® was adopted as the membrane barrier test by the OECD in 2006 (43).

Following the process of validation and adoption of *in vitro* test methods for skin corrosion, the European Union's classification and labeling system was modified with the introduction, in 2008, of the Globally Harmonized System for classification (GHS; UN, (44), by means of the EU Classification, Labeling, and Packaging Regulation (EU CLP; EU, (45). Briefly, if previously the corrosive category was divided into two subcategories in Europe (i.e., R34 and R35) according to the former EU Dangerous Substance Directive (EU DSD; EU, (46), the use of three corrosive subcategories, namely Cat. 1A, 1B, and 1C (which are optional according to the GHS), were implemented by the EU CLP. These three subcategories happened to be very similar to the three UN packaging groups I, II, and III (47).

During the original validation study both the EpiSkin™ and Corrositex® test methods had a prediction model developed, to distinguish the three UN packaging groups (UN PG I, II, III, and non-corrosive), which corresponded to the three EU CLP corrosive subcategories (Cat 1A, 1B, 1C, and non-corrosive, respectively). In addition, the TER method had a prediction model developed for distinguishing EU DSD R35, R34, and non-corrosives, which corresponded to the EU CLP/UN GHS Cat 1A, 1B+C, and non-corrosives, respectively. However, as the validation study was carried out during the late 1990s, the assays were evaluated for their ability to discriminate the EU DSD, and then the applicable non-corrosives versus R34 and R35 corrosive categories (or non-corrosives versus UN GHS Cat 1B+C and 1A), but it did not report on their predictive capacity to distinguish between UN PG II and III (i.e., between the EU CLP/ UN GHS Cat. 1B and 1C). A description will be given below on each of the validated and adopted assays for skin corrosion, with particular focus on their applicability for regulatory and industrial purposes.

The rat skin TER test uses excised rat skin as a test system and its electrical resistance as an endpoint. Test materials are applied for up to 24 hours to the epidermal surfaces of the skin discs. Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the TER below a threshold level (41). For a rat TER, a cut-off value of 5kΩ has been selected. Generally, materials that are non-corrosive in animals, but are irritating or non-irritating, do not reduce the TER below this cut-off value. A dye-binding step is incorporated into the test procedure for confirmation testing of positive results in the TER, including values around 5kΩ. The dye-binding step determines whether the increase in ionic permeability is due to the physical destruction of the stratum corneum. The rat skin TER method has been shown to be predictive of corrosivity in the in vivo rabbit test (OECD Test Guideline 404; 2002 OECD, (48). The TER method can also be applied to excised human skin and some differences between human and rat skin have been reported (49). It allows distinguishing corrosive from non-corrosive test materials, but does not allow distinguishing the three EU CLP corrosive subcategories.

Human skin model tests use cell viability as an endpoint measured by reduction of MTT (50). The principle of the human skin

model assay is based on the hypothesis that corrosive chemicals are able to penetrate the stratum corneum by diffusion or erosion, and are cytotoxic to the underlying cell layers. Test materials are applied to the surface of the human skin model and corrosive materials are identified by their ability to produce a decrease in cell viability, below the defined threshold levels, at specified exposure periods (41). The OECD guideline also provides guidance on the general and functional properties required for a new similar skin model, in order for it to be suitable for use in the test, and reference chemicals suitable for testing the predictive ability of a similar new model. In the original validation study, the EpiSkin™ human skin model was found to be suitable for distinguishing between R35 (EU CLP/UN GHS Cat. 1A) and R34 (EU CLP/UN GHS Cat. 1B+C) for all chemical types tested (28,30). On the other hand the three similar skin models later validated based on catch-up studies, EpiDerm™, SkinEthic™, and EST-1000, made use of prediction models designed to distinguish corrosives from non-corrosives, but not necessarily to distinguish between the corrosive subcategories such as those implemented within the EU CLP regulation. As a consequence, the originally adopted EU B.40 bis guideline mentioned that, "the test protocol may provide an indication of the distinction between severe and less severe skin corrosives," however, it also stated that, "*it does not allow the subcategorization of corrosive substances as permitted in the GHS*" (45). Similarly, the OECD TG 431 guideline stated that human skin models were useful for the distinction between corrosive and non-corrosives test materials, but not for the subcategorization of corrosive substances according to the GHS (42).

The Corrositex® test employs penetration of test substances through a hydrogenated collagen matrix (biobarrier) and a supporting filter membrane. Following the validation study and review of available data by the ECVAM Scientific Advisory Committee (ESAC) it was concluded that the Corrositex® test was a scientifically validated test, but only for those acids, bases, and their derivatives, which met the technical requirements of the assay (51,53). Corrositex® was not adopted into the EU legislation, as the rat skin TER and human skin model tests, but it was accepted by the US Department of Transport (US DOT) and the OECD as test guideline 435 on an *In Vitro* Barrier Membrane Test, based on the Corrositex® test method (43). Among the validated and adopted in vitro assays for skin corrosion testing, it is the only test accepted by the OECD and recommended by the European Chemicals Agency (ECHA) to distinguish the three EU CLP/ GHS/ UN subcategories (43,54). However it is considered valid only for limited applicability of acids, bases, and acid derivatives. For all other in vitro skin corrosion test methods, the Skin Corrosion Category 1A will be applied in case of a corrosive outcome.

Irritation

As systemic reactions play a minor role in modulating the local skin toxicity potential of chemicals, in vitro systems, which are sufficiently complex to mimic human skin barrier and cell reactivity, have been considered as potential models to predict the skin irritation potential of substances and have been evaluated over the last decade (16). Following an extensive review of the existing in vitro systems and toxicological endpoints (55,56) an ECVAM prevalidation study was conducted during 1999–2000, where five promising in vitro methods were evaluated, that is, EpiDerm™, EPISKIN™, Prediskin™, the non-perfused pig ear model, and the in vitro mouse skin integrity function test (SIFT). The study concluded that although the reproducibility of the two human skin model tests (EpiDerm™ and EPISKIN™) and the SIFT test was acceptable, their predictive capacity needed further improvement (58). ECVAM and its task force on skin irritation, therefore, recommended optimization of the protocols and prediction models of the three assays (60). Subsequent refinements were made to the three assays so that the optimized test protocols and/or prediction models met the criteria for inclusion in a formal validation study (61,23,62,63,24).

The ECVAM skin irritation validation study (SIVS) then took place, with the aim of evaluating whether the EpiDerm™, EPISKIN™, and the SIFT assays were able to reliably discriminate the skin irritant from non-irritant chemicals, and as such, to replace the rabbit Draize test for skin irritation. Further to the outcome of the validation study (64) and an independent peer review, the EPISKIN™ assay was considered to be a reliable and relevant stand-alone test for predicting rabbit skin irritation, when the endpoint evaluated was MTT reduction, and to be used as a replacement for the Draize Skin Irritation Test (OECD TG 404), for the purposes of distinguishing between R38 skin irritating and no-label (non-skin irritating) test substances, according to the EU DSD. Moreover, the IL-1 endpoint was regarded as a useful adjunct to the MTT assay, as it had the potential to increase the sensitivity of the test, without reducing its specificity, so that this endpoint could be used to confirm the negatives obtained with the MTT endpoint (65). Moreover, the EpiDerm™ assay was considered to reliably identify skin irritants, due to its high specificity, and further improvements were recommended to increase its level of sensitivity (65). Following this ESAC statement, modifications of the EpiDerm™ assay were made leading to the EpiDerm™ Skin Irritation Test (SIT) modified protocol. Moreover, a similar assay, based on Reconstructed Human Epidermis (RhE) and the SkinEthic™ RHE test method, was proposed for skin irritation testing. Catch-up studies were carried out on these two assays to determine whether they met the requirements of the performance standards, as defined by ECVAM, for in vitro skin irritation testing. Following a review by ESAC, both the assays were endorsed as scientifically valid, for having met the criteria outlined in the performance standards, and were endorsed to have sufficient accuracy and reliability for prediction of EU DSD R38 skin irritating and no label (non-skin irritating) test substances compared with the validated EPISKIN™ assay, including the limitations associated with it (66).

With the EU adoption of the UN GHS by means of the CLP Regulation, the performances of all three test methods (EPISKIN™, modified EpiDerm™ EPI-200, and SkinEthic™ RHE) have been re-evaluated, taking into account the shift of the cut-off value for the classification of skin irritants (a cut-off of 2 for R38 classification versus a cut-off of 2.3 for the GHS Cat. 2). Such an evaluation has been seen to be satisfactory, so the scientific validity of the three test methods was extended to the EU CLP (GHS) classification system (67).

A Test Guideline on "In vitro Skin Irritation: Reconstructed Human Epidermis (RhE) Model" has been adopted on the validated assays and included in the EU Test Method Regulation (EU test method B.46; EU, (68), and at the OECD level as TG 439 (69). The RhE tests falling under these guidelines allow classifying substances as skin irritants according to GHS Cat. 2. However, it does not allow classification of substances as mild irritants

according to the optional GHS Cat. 3, nor does it provide adequate information on skin corrosion. Depending upon the member country or regional regulatory requirements, all non-category 2 chemicals may be considered as non-classified (non-category). Thus, regulatory requirements in member countries will decide if this test method will be used as a skin irritation replacement test (i.e., in the EU), as a screening test, or as part of a tiered testing strategy, in a weight-of-evidence approach. Within the EU and countries from the European Economic Area that adopt the EU legislation, such a test is considered a stand-alone replacement test within a testing strategy, in a weight-of-evidence approach, in agreement with the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) guidance on information requirements and chemical safety assessment (70).

SAFETY ASSESSMENT OF SUBSTANCES AND PREPARATIONS

In general, the use of sequential testing strategies is generally recommended for the assessment of skin corrosion and the irritation potential of chemicals. At the OECD level the recommended testing strategy is based, in a sequential order, on: Existing human and/or animal data, Structure-Activity Relationships, pH considerations, systemic toxicity via the dermal route, the use of validated and accepted in vitro tests for skin corrosion, the use of validated and accepted in vitro tests for skin irritation, and finally, stepwise animal testing (OECD TG 404, 2002). In this strategy a substance found to be corrosive or an irritant in vitro should not be tested in the Draize test. However, if a negative result is obtained with the in vitro assays for skin corrosion and irritation, further testing may be carried out depending on the regulatory requirements from the OECD member countries. The United Nations Globally Harmonized System for classification and labeling, proposes a similar strategy to the one recommended by the OECD (44), with the only difference being that a human patch test is suggested as the last step after an in vivo test, if the test material has been shown to be non-irritant and non-corrosive.

Within the European Union, the Endpoint Specific Guidance to the REACH Regulation also proposes a sequential strategy for skin irritation and/or corrosion (70). Even as some of the building blocks are similar to the ones recommended within the OECD TG 404 and UN GHS, this test strategy introduces new elements, such as, the use of weight-of-evidence analysis of all existing and relevant data, and the use of validated and accepted in vitro methods for the identification of non-irritants, in addition to the identification of irritants and corrosives, so that the in vivo test can be avoided. Further specific testing strategies have been proposed including the one from Macfarlane and co-authors (2009), who have proposed integrated strategies for the hazard and risk assessment of cosmetic ingredients. Here again the use of weight-of-evidence analysis is proposed, to evaluate all available data, such as, the physicochemical properties, literature, animal, in vitro, human, read-across, and Structure-Activity Relationship (SAR). Such evaluation is then followed by an in vitro test for skin corrosion and an in vitro test for skin irritation. No in vivo or human testing is used for hazard assessment. However, human testing is proposed for risk assessment.

The assessment of safety can indeed be considered in two separate parts: The risk to health from exposure to an individual substance, which is largely related to occupational health, and the risk to consumer and occupational health from exposure to preparations, for example, personal care products, medicaments, cleaning products, and so on. In the former case, occupational dermatitis is known to arise from exposure to single substances, although typically, this is accidental exposure to corrosive materials (9). The validation studies on skin corrosion and irritation have been carried out mainly using individual chemical substances, so that little information is available on the usefulness of the validated and adopted in vitro methods, to test preparations, mixtures, and/or dilutions. However, testing of preparations with in vitro test methods may be useful for several purposes, including within the framework of EU CLP, where in vitro testing of preparations may be required in order to obtain accurate classification and avoid unnecessary animal testing (45). In this case, a recommended strategy to identify the most suitable assays for a specific product class include: (i) Start by evaluating the usefulness of a validated test protocol, (ii) build up a large database with the product class, (iii) identify benchmark materials close to the classification border backed by robust in vivo data, preferably both animal and human, and (iv) optimize the test protocol and/or prediction model if appropriate (71).

Furthermore, testing of dilutions could be useful to estimate the Derived No-Effect Levels on skin irritation/corrosion for a given test material based on dose–response information. Figure 38.1 presents, in generic form, the type of assessment that may be considered using in vitro models. Some simple rules for the development and/or identification of the best assays for a specific purpose are necessary. The in vitro test must be preferentially a validated or standardized model for which there is considerable experience in the institution; the substances/products to be tested must be of similar type; there must be knowledge that the relative irritating ability of these substances/products is clinically reflected by the results from the in vitro test. In such a situation, it may be relatively straightforward to simply compare the dose response curve for a known material with an unknown substance/product. The dose response displayed in Figure 38.1 displays an increasing response (the values are arbitrary), which suddenly falls as cell death dominates at the highest concentration. However, it can be seen that the test material behaves similarly to the known control, albeit giving a slightly lower degree of response. In this situation, one might readily conclude that the test material, whether a

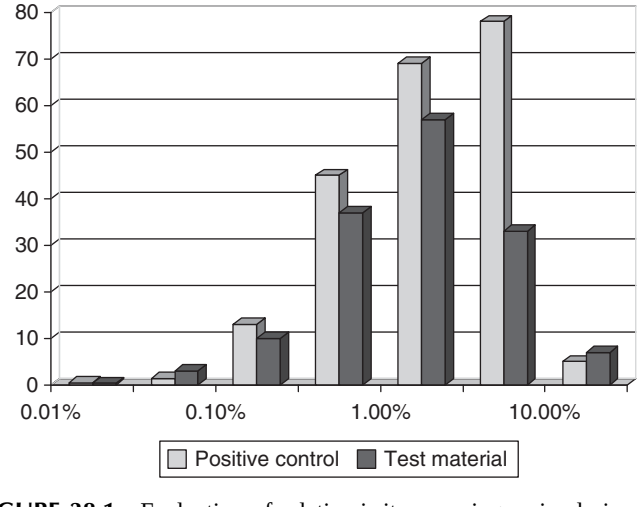

FIGURE 38.1 Evaluation of relative irritancy using a simple in vitro procedure.

substance or a product, is likely to be just a little less irritating to skin. Such a conclusion is entirely dependent on the quality of the historic evaluation of the in vitro assay against the clinical data. Another example is shown by Spiekstra and co-workers (2009), who developed a protocol based on reconstructed epidermis models, which is able to determine the skin irritant potency of chemicals and not only distinguish irritants from non-irritants.

FUTURE PROSPECTS

A workshop was organized in 2010 by the Swiss Federal Office of Public Health in order to develop harmonized recommendations on the use of in vitro skin corrosion and irritation data for regulatory assessment purposes. The workshop comprised major stakeholders involved in the process from in vitro testing to risk assessment including regulators, ECVAM, industry, Contract Research Organizations (CROs), and test developers (71). Among the recommendations made during the workshop, an important one was to give more flexibility to the current proposed strategy sequences for the testing of chemicals (e.g., OECD, (48); ECHA, (72); UN, (44), so that the current recommended testing strategies are not necessarily interpreted as a prescribed order of tests (71). Indeed, being a supplement to the TG, the proposed OECD test strategy does not fall within the Mutual Acceptance of Data, and hence, can be considered as recommended, but not necessarily binding to the OECD member countries. It is foreseen that based on the amount and usefulness of information available on the test material, different options may be undertaken: (1) In case sufficient and adequate information for classification are available: classify and label with no testing needed, (2) in case the available information might be useful, but not sufficient for classification and labeling: make use of a testing strategy tailored to specific test materials (composed from the set of options proposed in the recommended strategies), and (3) in case existing information is not available or not useful: use the default recommended testing sequence in order to generate new data. For sectors where applicability of the recommended strategies is questionable (e.g., pesticides), it is suggested to collect information and design and/or construct specific strategies suitable for their specific purpose(s), based on a large database of the material in question.

In 2010, the OECD started an initiative that may address some of the issues listed above. The aim is to develop a guidance document on skin irritation and corrosion, and/or develop recommendations for potential revisions/deletions/merging of the existing in vivo and in vitro skin irritation and corrosion guidelines, that is, OECD TG 404, 430, 431, 435, and 439. Some of the items proposed for discussions include: How the TGs are actually used by industry and regulatory authorities, what the limitations and strengths of the TGs and individual test methods are, which chemical classes should be covered, whether mixtures and preparations should be included, and the identification of false negative corrosives by the skin irritation test method. It is hoped that if a direct replacement of the TG 404 is not possible, the newly generated information may be able to demonstrate the limitations and benefits of all the concerned OECD TG, and allow outlining recommendations on how to achieve replacement of the in vivo test, if considered possible.

Another consideration made was that a full replacement for subcategorization of two and/or three sub-classes of corrosivity was necessary for transportation of chemicals and mixtures and to avoid unnecessary animal testing for this purpose. For this reason it was recommended to update the OECD TG 431, in order to indicate the usefulness of the EpiSkin™ assay to distinguish Cat.1A from Cat. 1B, based on the results from the ECVAM validation study (28,30). Furthermore, additional studies were suggested to, (1) investigate the EpiSkin™ RhE predictive capacity to subcategorize corrosive chemicals as Cat. 1C, based on its current protocol and (2) to optimize and/or investigate the usefulness of the EpiDerm™, SkinEthic™, and EST-1000 RhE validated protocols for the subcategorization of corrosive chemicals, according to the three EU CLP corrosive subcategories 1A, 1B, and 1C.

Despite the considerable progress achieved in the area of in vitro skin irritation and corrosion, the current in vitro strategies still very largely focus on classification of the intrinsic hazards of chemicals, rather than permitting the assessment of risk to human health. For such a purpose, the development of optimized in vitro tests, as described in the previous chapter, may be of help in order to obtain, for example, a more continuously distributed endpoint measurement. Also important in this respect is the assessment of the propensity of a formulation to cause skin irritation as a consequence of repeated exposure. Assessment of the ability of products to cause skin irritation in response to repeated exposure forms the subject of a number of reviews (73,74), and typically involves clinical studies and will not be repeated here. However, only very limited efforts to model the repeated responses as well as determine the potency of chemicals in vitro have been reported (75,76).

Overall, there is a need to move from rigid integrated testing strategy guidelines to more flexible strategies tailored to specific purposes and/or use sectors, even though such a ‘tailor-made approach’ may require further work. Sharing of information between individual companies may be of help to build up robust databases, better understand the mechanisms of action of specific product categories as well as the applicability and limitations of the currently accepted in vitro test methods and strategies for specific purposes and uses.

IN VITRO PHOTOTOXICITY TESTING — BACKGROUND

Safety tests for phototoxicity have historically used animal models (reviewed by Lambert et al., (77), largely involving guinea pigs. However, there has been no standardization of procedures and it can be difficult to compare results from different laboratories Maurer, (78,80)). Draft proposals for OECD Guidelines for phototoxicity testing in vivo were considered in 1991 and 1995 (81,82). However, these proposals never progressed, largely because of an unwillingness to define further animal models, because of pressure from consumers and legislators, notably in Europe, to introduce alternative models in vitro. In addition, developments in cell culture and bioanalytical techniques stimulated renewed interest in the use of in vitro models for safety hazard evaluation (83). A Task Force on phototoxicity testing in vitro was set up on the initiative of the European Cosmetic Toiletry and Perfumery Association (COLIPA), which was joined in a joint project by the EU, through the ECVAM. In vitro phototoxicity testing was the topic of the second ECVAM workshop that was organized in 1993, in collaboration with the COLIPA task force, on phototoxicity testing (80). The aim of this workshop was to plan a validation study on the most promising in vitro phototoxicity tests and to identify an optimum set of test chemicals, based on high quality in vivo data on humans.

A list of high quality data from standardized human photopatch testing for both acute phototoxicity and photoallergy was made available. A total of three studies were subsequently conducted to validate the 3T3 Neutral Red Uptake phototoxicity test (NRU PT), which has now been accepted for regulatory purposes within the European Union and by the OECD (see below). A second ECVAM workshop on in vitro phototoxicity testing was held in 1999. The status of testing methods and their development since the first ECVAM Workshop was discussed and a report published, which focused on human photopatch testing, in vitro photogenotoxicity, in vitro methods for assessing the photoallergy potential of chemicals, the application of 3-D human skin models for in vitro phototoxicity testing, and general testing strategies covering all aspects of in vitro phototoxicity (84).

VALIDATION STATUS OF IN VITRO PHOTOTOXICITY ASSAYS

Photoirritation

An in vitro photocytotoxicity model, using mouse 3T3 fibroblasts, with neutral red uptake as the endpoint (the 3T3 NRU PT) was first developed and prevalidation started in 1992. This test is based on a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of simulated solar light (UVA/visible spectrum). Cytotoxicity is expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red when measured 24 hours after treatment with the test chemical. Substances identified by this test are likely to be phototoxic following systemic application and distribution to the skin, or after topical application. Prevalidation was carried out by eight laboratories in a non-blind trial using 20 chemicals (11 phototoxic and nine non-phototoxic), and they developed a prediction model using a photoirritation factor (PIF: EC_{50} value -UV/EC_{50} value +UV) to discriminate between the positive and negative chemicals (85). Using a cut-off value of PIF = 5, all of the test chemicals were correctly classified in the 3T3 NRU PT. Similar results were obtained in an independent study conducted at the Hatano Research Institute, in Japan, in 1994, using the same test protocol and the same chemicals (Wakuri et al., 1994). The following formal blind validation trial using 30 test chemicals (25 phototoxic and five non-phototoxic) demonstrated that the test was reproducible in the nine participating laboratories, and that correlation between in vitro and in vivo phototoxic potential was very high, with all phototoxic chemicals being correctly identified (86). At the request of the Scientific Committee of Cosmetics and Non-Food Products (SCCNFP), the expert advisory committee on cosmetics, a set of the most commonly used ultraviolet (UV)-filter chemicals, which are not phototoxic in vivo and poorly soluble in water, plus a set of known phototoxic chemicals, were tested in a further blind trial with the 3T3 NRU PT (20 chemicals in four laboratories). The test was found to correctly assess the phototoxic potential of modern UV filter chemicals (87). The NRU PT protocol could also be used with human keratinocytes, as demonstrated in a blind study with the chemicals of the EU/COLIPA validation study and the UV-filter study (88). The 3T3 NRU PT, because of its successful validation, was officially accepted by the European Commission and the EU Member States into Annex V of the EU Council Directive 67/548/EEC for the classification and labeling of hazardous chemicals (90). An OECD guideline for the NRU PT (OECD Test Guideline 432) was accepted and published in 2004 (42). For TG432, a modified prediction model was developed based on the results of the validation trials; a test substance with a PIF <2 predicts: "No phototoxicity", a PIF >2 and <5 predicts: "probable phototoxicity" and a PIF>5 predicts: "phototoxicity". TG432 also reflects the conclusions of the validation trials that false positive results may be obtained at high test concentrations, and recommends a maximum test concentration of 1000 µg/mL.

Although the NRU PT is the only validated in vitro test for photoirritation, additional tests also exist (93). A photo-red blood cell hemolysis test (Photo-RBC) has undergone prevalidation evaluation using the 30 chemicals used in the NRU PT validation (Pape et al., 2000). In the protocol for the Photo-RBC test, two endpoints are determined in the erythrocytes, namely, photohemolysis, as a measure of primary type II photoreactions, and methaemoglob information(met-Hb), as a measure of type I photoreactions. There have been some problems with inter-laboratory transferability of the protocol, but the overall conclusion of the prevalidation study is that the Photo-RBC test can be performed reproducibly and it provides relevant mechanistic information on photoreactions for use within a wider testing strategy. RBCs are also resistant to UVB, which enables exposure to the entire solar spectrum, compared to the NRU PT. Yeast (Saccharomyces cerevisiae), is also relatively insensitive to both sunlight and prolonged exposure to test materials, which has also led to its proposal for use in both phototoxicity and photogenotoxicity tests (using mutant strains deficient in DNA repair pathways). These tests have not undergone any validation activity, but have been reviewed by the second ECVAM phototoxicity workshop (84).

The NRU PT has been demonstrated to detect the phototoxic potential of both strong and weak phototoxins, irrespective of their aqueous solubility (87). In the case of negative responses, however, there may be some uncertainty, as the chemical can be tested only at low concentration because of lack of aqueous solubility. Assays using 3-D reconstructed human skin models can help address this. 3-D skin models allow the application of various types of test materials (undiluted and using both aqueous and organic solvents) and preparations to their surface, and therefore, have fewer solubility problems. 3-D skin models are considerably less sensitive to UVB than monolayer cells (21,94). This allows the possibility of using light source emitting, in addition to UVA, a greater proportion of UVB compared with sources used for monolayer cell models, thus further mimicking sunlight (95). Skin2™ (a currently unavailable commercial model), was originally reported to identify phototoxic hazard potential (97–99) in a similar manner to the NRU PT, with evaluation of phototoxic hazard potential via an MTT viability assay (50). Similar protocols have since been successfully transferred to currently available 3-D skin models, such as EpiDerm™, EpiSkin™, and SkinEthic™ (21,22,100–104,95). EpiDerm™ has also undergone evaluation in an ECVAM prevalidation study, which gave good predictions in the three laboratories using ten chemicals (105). Being relatively expensive these models are not suitable to use in a screen to predict a phototoxic hazard, but are useful in the further evaluation of materials for topical application, in a testing strategy.

Photoallergy

The results of the validation trial of the NRU PT showed that the model detected both photoirritant and photoallergic chemicals, however, there are no validated in vitro tests for photoallergy

per se. Photoallergy is a delayed type of hypersensitivity, with an essential requirement for UV radiation (106). Photochemical binding of photoallergens to a protein is widely accepted as the initial step of the photoallergenic process and has been proposed as a test for potential photoallergenicity (107,108). Photoirritants may also photobind to a protein, but in this case other photochemical reactions are expected to be more significant and photo-oxidation of histidine has been proposed to identify the photo-oxidizing potential, which may lead to photoirritancy (109). Efficient photo-oxidisers may be considered photoirritant rather than photoallergic. A photobinding assay using binding to human serum albumin, in conjunction with a test of photo-oxidation of histidine, was used to test the 30 chemicals used in the NRU PT validation trial (110). Six of the seven photoallergens were identified by the photobinding assay. Most photoirritants also caused photomodification of the protein, but 11 (out of 17) also photo-oxidized histidine efficiently and so were classified as photoirritants. Four photoirritants remained falsely predicted as photoallergens. Two photoirritants were negative for both photomodification of the protein and for histidine photo-oxidation. Four chemicals negative in vivo were negative in vitro. The two remaining chemicals could not be classified because of unclear data both in vivo and in vitro. Therefore, there was good detection of photoallergens. Differentiation between photoallergens and phototoxins was seen, but not achieved in all cases.

SAFETY ASSESSMENT OF SUBSTANCES AND PREPARATIONS

Where substances are intended for use in products that are either intended for skin application or which may come into contact with the skin, it is necessary to carry out an assessment of the potential phototoxic hazard. Figure 38.2 shows a stepwise testing strategy suitable for this purpose. An essential requirement for phototoxicity is the absorption of light by a test material; the initial assay is the measurement of a UV/visible absorption spectrum, to identify the absorption at relevant wavelengths (>300 nm). The OECD guidelines state that if the molar extinction/absorption coefficient of a chemical is less than 10 liters x mol-1 x cm-1 the chemical is unlikely to be photoreactive and need not be tested in the 3T3 NRU phototoxicity test or any other biological test for adverse photochemical effects (112,42). Another similar practical cut-off for absorption is, if the absorption of a 1% solution using a 1 cm path length (A1%1 cm) is less than 1.0, then similarly this would not be considered as significant (109,110). This is useful where the molecular weight of a substance is not known or for the consideration of extracts/mixtures. If a substance demonstrates significant UV/visible light absorbance, then it may have potential for phototoxicity and must be tested. The primary test of choice would be the validated NRU PT, which has the potential to detect photoirritants and also most photoallergens and photogenotoxins. If a substance is negative in the NRU PT, then this is good evidence that it does not have phototoxic potential and should not require further testing (Spielmann et al., 1998a and 1998b). Examples have been published on the testing of several types of ingredients such as surfactants (113), fragrances (114), and essential oils (115).

In risk assessment practice, a further confirmatory test may be desired to add to the weight of evidence demonstrating the absence of a hazard, prior to marketing the substance. Further testing would also be advisable where a substance gives a borderline result of probable phototoxicity in the NRU PT. In this case, further testing using a 3-D skin model assay would be recommended (95). The advantages of 3-D skin models in providing a system more similar to the human skin in vivo are given above. Toxicity (and phototoxicity) to human skin is affected by penetration rates, through the stratum corneum. In general, the penetration rates of substances through in vitro skin models, where measured, are greater than that of human skin (116–120). Therefore, 3-D skin models would be considered as more sensitive to insult than human skin per se and the lack of phototoxicity of a substance in such a model is good evidence that it would not present a phototoxic hazard to human skin in vivo. An example of the use of a 3-D skin model assay, to further investigate borderline results in the NRU PT for a personal product ingredient, has been published by Jones and co-workers; in this study the material was found to be

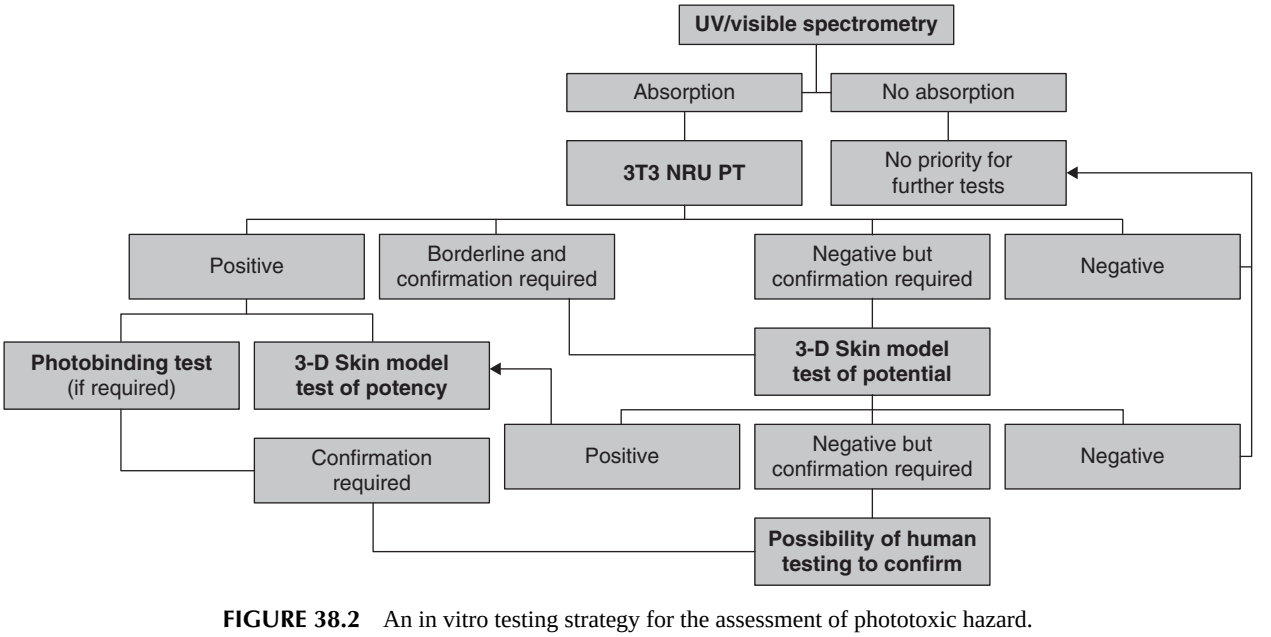

FIGURE 38.2 An in vitro testing strategy for the assessment of phototoxic hazard.

non-phototoxic to the skin model in vitro (121). Alternatively, a borderline result may be confirmed as positive by the 3-D skin model.

A positive result for a substance in the NRU PT (or a 3-D skin model) provides evidence of a possible phototoxic hazard (which would feed into the overall risk assessment of the substance. If further information on the nature of the hazard is required then a photobinding test for photoallergy may be carried out. At this stage, further information can also be obtained by carrying out a dose–response for photoxicity in a 3-D skin model. This could give information on the possible potency, by comparison with the intended use levels, and in particular by comparison with a phototoxin of known in vivo potency (bearing in mind the probable greater sensitivity of the skin models than human skin in vivo). The final step in the testing strategy, is the possibility of testing in a human clinical trial, provided sufficient information has been obtained for ethical testing, a situation that seems unlikely, except for the essential novel pharmaceutical preparations; for consumer personal care and household products, evidence of photopositivity is most likely to lead to cessation of product development rather than clinical testing. However, it should be noted that 3-D skin models also lend themselves to the testing of formulations, as they allow application to the stratum corneum surface (104,93). It has been demonstrated that formulations spiked with a known phototoxin can be identified using a 3-D skin phototoxicity test (95). A 3-D skin model phototoxicity assay could therefore be useful as an interim step, as part of the risk assessment process prior to any human testing.

FUTURE PROSPECTS

In October 2010, ECVAM and the European Federation of Pharmaceutical Industries and Associations (EFPIA) jointly organized a "workshop on the 3T3 NRU Phototoxicity Test: Practical Experience and Implications for Phototoxicity Testing" (122), to discuss why several pharmaceutical companies were encountering an unexpected high percentage of positive results with the 3T3 NRU PT (about 50%), with a majority of in vitro positives (about 80%) turning to be negative in animals or humans (Lynch and Wilcox, 2009). Thirty-five experts from the academia, regulatory authorities, and cosmetics and pharmaceutical industry, participated in this workshop to contribute with their experiences in the field of in vitro photo safety assessment. The experts concluded that 3T3 NRU PT is a hazard-based assay, with a high level of sensitivity, thus being a highly relevant and accepted test to correctly identify non-phototoxic materials. However, positive results in the 3T3 NRU PT often do not translate into a clinical phototoxicity risk. Therefore, it was concluded that it is important to find ways to improve the specificity of this assay in order to avoid unnecessary testing in vivo, but without affecting its high sensitivity. During the workshop different ways to improve the predictivity of the assay were discussed, with the following main recommendations being made:

1. Consider testing only compounds showing a Molar Extinction Coefficient (MEC) > 1000 L/mol/cm. Absorption is a precondition for phototoxicity, that is, if a chemical does not absorb light it will not be phototoxic. Therefore, MEC could be used as a screen to decide if phototoxicity testing is required (123).
2. Limit the top concentration of the test material under irradiation to 100 g/mL, instead of 1000 g/mL recommended in OECD TG 432, as most phototoxins are positive in the 3T3 NRU PT in the concentration range of 0.01–10 μg/mL. Accordingly, in the original 3T3 NRU PT validation study, a safety factor of 10 was considered suitable and the maximum concentration proposed was 100 g/mL. Indeed, the maximum concentration currently recommended in OECD TG 432 is generally much higher than the likely clinical plasma exposure or exposure to the tissue of relevance for determination of in vivo phototoxicity (122).
3. Depending on a more systematic analysis of how phototoxicity in vivo is related to PIF/MPE values from data collected from the industry, consider modifying the criteria used to identify "positives" in the 3T3 NRU PT. Thus, it was proposed to apply PIF <5 or MPE <0.15 thresholds for "negative" results (non-phototoxic) more generally (according to validation data), rather than PIF <2 or MPE <0.1. Importantly, it was concluded that it may be necessary to develop different protocols and/or prediction models for topical and non-topical (systemic) materials.

The experts attending the workshop also agreed on the following recommendations to help improve the interpretation of 3T3 NRU PT results and their use in risk assessment:

1. Positive PIF/MPE values should not prevent further development. Instead, follow-up testing in a defined strategy should be performed, to obtain data with models that better reflect the human situation, such as 3-D skin models.
2. Positive PIF/MPE values obtained at higher concentration of the compound (high IC_{50}s under irradiation), are less likely to correlate with relevant human in vivo/clinical signs of phototoxicity. In case human exposure levels are known, comparison with IC_{50}s under irradiation might help estimate the clinical relevance.

Finally, in vitro alternatives to the 3T3 NRU PT were discussed during the workshop. In particular, Reconstructed Human Tissue models were suggested to be reliably used for the identification of the phototoxic hazard for topically applied compounds, as a second tier to verify 3T3 NRU PT positive results, in order to reduce the number of false positives. Reconstructed human 3-D skin models present several advantages when compared with the 3T3 NRU PT, such as, the presence of a skin barrier similar to in vivo human skin, the presence of metabolic competence, the possibility of topical exposure and absence of concerns for chemicals with limited solubility, and the ability to provide potency information.

In 2011, the validation of a reactive oxygen species (ROS) assay for detection of the phototoxicity potential of chemicals through UV irradiation was initiated by the Japanese Center for the Validation of Alternative Methods () and the Japan Pharmaceutical Manufacturers Association. The main objective of this study was to assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of the ROS assay, with the final goal of incorporating it in a tiered

strategy for photo safety assessment, according to the framework defined by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). As most phototoxic compounds have the ability to generate ROS under photoirradiation, the determination of ROS could be an effective predictive model of phototoxic potential (124,125). Moreover, the ROS assay is considerably cheaper to perform that the 3T3 NRU PT, and it may be able to detect UVB absorber phototoxins that are negative in the 3T3 NRU PT, even though it may suffer from an even lower specificity than the 3T3 NRU PT. Therefore, the ROS assay is foreseen as a possible tier 1 in a photo safety assessment testing strategy, triggering further testing in case of a negative result. However, at the time of writing, the results are not available for further commentary to be possible.

Ultimately, risk assessment for photo endpoints remains challenging. Clearly, it would be unwise to treat a positive photosensitization result with anything other than a good deal of caution. The absence of dose response information means that determination of the relative photo-sensitization potency is difficult (unless at the extreme ends of the spectrum). What is required is that the result with the new substance be compared with the information in the same test model, and with the results from a range of known photosensitisers, which will thereby permit a "calibration" of the assay. For this purpose, materials such as tetrachlorosalicylanilide and musk ambrette are of limited use; what is really needed are photosensitizers whose use levels and types of human exposure are understood and where the extent of photosensitization induced in humans is at a level that is known and well tolerated (by consumers, regulators, and dermatologists). An example of this could be butylmethoxydibenzoylmethane (Parsol 1789), which is a valuable UV-A filter sunscreen chemical, widely used, known to be a photosensitizer, but is associated with only a very low and acceptable rate of human positives in diagnostic photopatch testing. The risk assessment strategy that then flows from this is that, if the new substance gives positive photosensitization results, similar to the benchmark and at similar concentrations, then human exposure at similar levels and in similar product types might reasonably be expected to yield the same sort of level of induction of photoallergy in humans. Of course, given the uncertainties associated with in vivo tests and extrapolation to man, a certain degree of caution should be adopted in such a risk assessment, perhaps via incorporation of an additional safety margin. It would also be appropriate to ensure that post market surveillance was actively undertaken (Jowsey, 2006). Similar risk assessment strategies might usefully be deployed for other photo endpoints.

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39 The local lymph node assay

David A. Basketter, Ian Kimber, Rebecca J. Dearman, Cindy A. Ryan, and G Frank Gerberick

INTRODUCTION

The acquisition of skin sensitization is dependent upon the initiation of an immune response and specifically a cell-mediated immune response. The relevant events and processes can be summarized briefly as follows: Sensitization is induced when an inherently susceptible individual is exposed topically to an appropriate and sufficient amount of contact allergen. Following entry into the skin, the chemical allergen either directly, or indirectly, associates with the protein and is recognized and internalized by cutaneous dendritic cells (DCs) including epidermal Langerhans cells (LCs) and dermal DCs. It is now clear that DCs play several pivotal roles in the generation and regulation of cutaneous immune responses and the induction of skin sensitization. For sensitization, their most important responsibility is the transport of antigen, via the afferent lymphatics, to the draining lymph nodes. During this migration from the skin, DC are subjected to a functional maturation, and as a result, by the time they arrive at the lymph nodes they have acquired the characteristics of the immunostimulatory antigen presenting cells (1,2). In the lymph nodes, the antigen is presented to T lymphocytes and the responsive cells become activated and are stimulated to divide and differentiate. Cell division results in a selective clonal expansion of allergen-responsive T lymphocytes; this quantitative increase in specific T lymphocytes represents the cellular basis for sensitization and immunological memory. If the now sensitized subject is exposed again to the same chemical, at the same or a different site, then this expanded population of specific T lymphocytes will recognize and respond to the allergen in the skin and trigger an accelerated and more aggressive secondary immune response, which in turn causes cutaneous inflammation that is recognized clinically as allergic contact dermatitis. The molecular and cellular mechanisms that result in the induction and elicitation of contact allergy have been reviewed extensively elsewhere (3–5). For the purpose of this article it is sufficient to state that the ability of chemical allergens to stimulate lymph node cell (LNC) proliferative responses is the event upon which the local lymph node assay (LLNA) is founded.

There have been a number of review articles that have considered various aspects of the LLNA (6–22). The purposes here are to review the development and subsequent evaluation and validation of the LLNA, and to examine the use of this method for hazard identification, potency evaluation, and risk assessment.

DEVELOPMENT OF THE LLNA

Based on an appreciation of the events induced during skin sensitization, the initial objective was to determine whether a method for hazard identification could be developed in mice that might be used as a viable alternative to the then favored guinea pig assays. In contrast to those guinea pig methods (in which activity is measured as a function of challenge-induced cutaneous reactions in previously sensitized animals), the strategy adopted was to focus on events induced during the induction phase of skin sensitization, and in particular, on changes provoked in lymph nodes draining the site of exposure. Several parameters of lymph node activation could be viewed as legitimate potential correlates of skin sensitization, including increases in lymph node weight and cellularity, the appearance of pyroninophilic cells, and the stimulation of LNC turnover (23,24). Preliminary investigations revealed, however, that of these, the induction of LNC proliferation represented the most sensitive and most selective marker of the skin sensitizing activity. In the initial studies, proliferative activity had been measured in vitro during culture of the draining LNC with [^{3}H] thymidine (^{3}H-TdR) (23,24). However, one important development was to measure lymph node hyperplastic responses in situ instead (25,26). This adaptation not only provided a more holistic and more sensitive assessment of cell turnover by LNC, it also served to obviate the need for tissue culture (with consequential logistic advantages). It is this form of the LLNA that has been the subject of extensive evaluations, and that has been subsequently validated.

The basic protocol for the LLNA has been described in detail elsewhere (16,27–30), but can be summarized briefly as follows: Mice of CBA strain are used. Groups of mice receive topical applications of various concentrations of the test chemical (or of the relevant vehicle control) daily for three consecutive days. Recommendations regarding suitable test concentrations are available elsewhere (30). For the purpose of hazard identification, it may be considered desirable to select the highest recommended test concentrations. In practice, however, this is not always possible. Concerns regarding local or systemic toxicity, and/or poor solubility, may dictate a more conservative approach.

Several vehicles may be used, and again those usually favored are considered elsewhere (8,31,15,30,32). Decisions regarding the choice of vehicle (in the context of hazard identification at least) are reached usually on the basis of suitability for topical application and the solubility of the test material. It is relevant to mention here that the vehicle in which a chemical allergen is encountered on skin surfaces can have an impact on the extent to which skin sensitization is acquired, and on the vigor of responses in the LLNA (8,33–38). There is no doubt that the vehicle matrix also influences the elicitation of responses in other methods, for the identification of contact allergens. Although vehicle effects have, in practice, little or no impact on the performance of the LLNA, in

the context of hazard identification, they are (quite rightly) of more significance when considering LLNA dose responses for the purposes of potency and risk assessment. This issue will be addressed later in this chapter.

Five days following the initiation of exposure, mice receive an intravenous injection of ^{3}H-TdR. nimals are sacrificed five hours later and the draining auricular lymph nodes excised. These are either pooled for each experimental group, or are alternatively pooled on a per-animal basis. Single cell suspensions of LNC are prepared and the cells washed and suspended in trichloroacetic acid (TCA) for at least 12 hours, at 4°C. The precipitates are suspended in TCA and transferred to an appropriate scintillation fluid. The incorporation by the draining LNC of ^{3}H-TdR is measured by scintillation counting and recorded as mean disintegrations per minute (dpm) for each experimental group or for each animal. In those instances where it is deemed appropriate to include a positive control within the test protocol, it is recommended that hexyl cinnamic aldehyde (HCA) be used for this purpose (39–41).

For each concentration of test material, a stimulation index (SI) is calculated, using as the comparator the (disintegrations per minute; dpm) value derived from the concurrent vehicle control. Skin sensitizers are defined as those chemicals that at one or more test concentrations are able to induce an SI of 3 or greater, relative to the concurrent vehicle controls. It must be recognized that the original decision to use an SI value of 3 as the criterion for a positive response in the LLNA was arbitrary, the choice being made on the basis of experience with a range of chemical allergens and non-sensitizing chemicals. However, it would appear that the decision was correct, as continued experience has revealed that in practice an SI of 3 appears to provide an accurate identification of skin sensitizing chemicals. Moreover, a retrospective analysis of results obtained with some 134 chemicals in the LLNA was reported in 1999 (42). The data were subjected to a rigorous mathematical assessment using Receiver Operator Characteristic (ROC) curves. The conclusion drawn from these analyses was that an SI value of 3 provides an appropriate criterion for the identification of contact allergens (42). Despite the proven value of an SI of 3 for hazard identification, some flexibility is appropriate when interpreting LLNA data. It has been recommended previously (30) that the characteristics of dose-response relationships and other factors must be taken into account. Thus, for instance, if a test chemical were to display a dose-related increase in LNC proliferative activity that just failed to achieve an SI of 3 at the highest concentration, then it would, in most circumstances, be inappropriate to conclude that the material lacked any potential to cause skin sensitization. In such cases it would be prudent, if possible, to conduct a repeat analysis, using, if possible, higher concentrations of the test chemical and/or a different vehicle.

A summary of the conduct of the standard LLNA is illustrated in Figure 39.1. Comprehensive lists of substances tested in the assay can be found in two review publications (43,44).

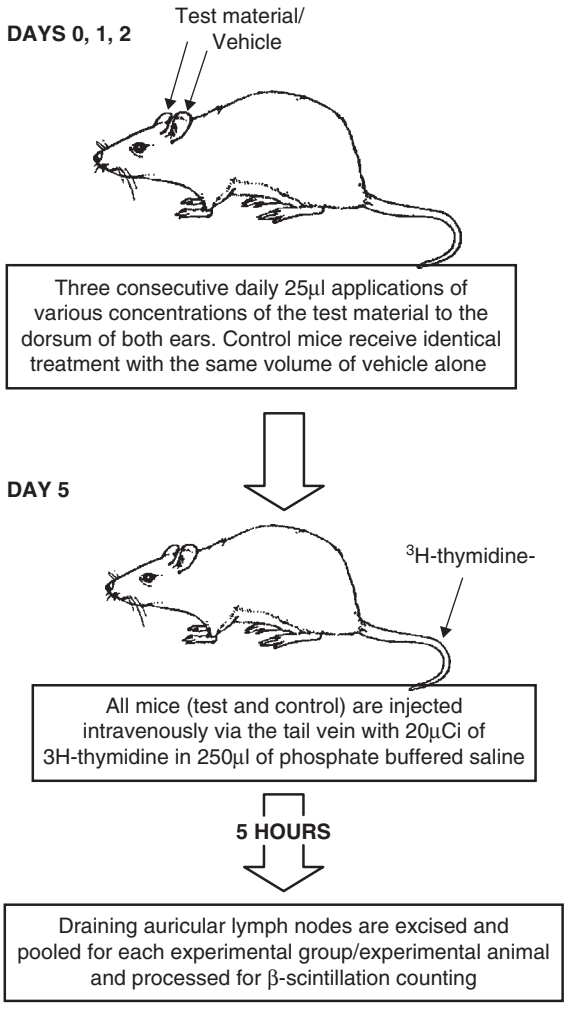

FIGURE 39.1 Conduct of the standard local lymph node assay.

EVALUATION AND VALIDATION

The LLNA was developed initially as a method for hazard identification. Although it is now clear that the LLNA is also of considerable utility in the determination of relative potency and in the risk-assessment process, it is for the purposes of hazard identification that the assay has been formally validated. That process of evaluation and validation is described here. Use of the LLNA for potency determination and risk assessment is considered later.

The LLNA has been evaluated extensively in both national and international inter-laboratory collaborative trials (45,46,30,47–51), and has been the subject of searching comparisons with guinea pig–predictive test methods and human sensitization data (52–54,45,39,55,20,56). Collectively, these investigations comprised analyses of a wide variety of chemicals. In addition, however, more discrete investigations of specific groups of materials have been conducted using either the standard LLNA or modifications of it. Among these are studies on biocides (57–59), fragrance materials and materials used in personal care products (60,38), metal salts (61), rubber chemicals (62), petrochemicals (63), dyes (64,65)) and chemical mutagens, and rodent carcinogens (66–68). On the basis of these investigations and additional practical experience gained from the use of the method, the conclusion drawn was that the LLNA represented a viable alternative to guinea pig tests for the identification of contact allergens (46,69,18).

Against this background the LLNA was submitted, in 1998, for consideration, by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), an organization established in the USA by 14 Federal regulatory and research agencies, to harmonize the development, validation, and acceptance of new toxicological test methods (70). A peer review panel

was appointed by the ICCVAM and after intensive scrutiny of the method it concluded that compared with other predictive tests, the LLNA offered advantages with respect to animal welfare (specifically in terms of refinement and reduction). The panel also recommended that the LLNA could be used as a stand-alone alternative for the purposes of hazard identification, subject to the implementation of certain protocol modifications. These proposed modifications included consideration of selection of mouse strain, the individual identification of mice, analysis of body weight changes, the use of statistical analyses, and the incorporation of a concurrent positive control (71,72,70,73). The utility and application of these modifications has been the subject of a detailed commentary (74), and a similar analysis here would be beyond the scope of this chapter. However, the important point is that the LLNA was subjected to rigorous independent scrutiny and validated by the ICCVAM as an appropriate method for hazard identification. There soon followed a similar endorsement by the European Center for the Validation of Alternative Methods (ECVAM) (75). In the light of these developments the current regulatory status of the LLNA is outlined briefly in the next section.

Although the LLNA has been shown in the context of the validation exercises summarized above, to have the levels of sensitivity, selectivity, and overall accuracy comparable with, or better than, the commonly used guinea pig tests, it nevertheless raises questions about specific issues relating to test performance. Among these are the ability of the assay to detect metal allergens (and in particular nickel) and the prevalence of false positive responses.

Nickel is a common human allergen. Although modest responses to nickel chloride and nickel sulfate can be induced in mice (27,76,32), the consensus is that nickel salts usually fail to test positive in the LLNA (20). However, it has also often proven difficult to elicit responses to nickel salts in guinea pig tests (77–79). The ability of the LLNA to detect metal salts that are known to be implicated in allergic contact dermatitis has now been examined systematically. Thirteen metal salts were studied, of which eight were considered to be contact allergens. The remaining five were considered not to cause skin sensitization. With the exception of nickel chloride, which is clearly a special case (80), all known allergens (tin chloride, cobalt chloride, mercuric chloride, ammonium tetrachloroplatinate, potassium dichromate, beryllium sulfate, and gold chloride) were found to induce positive responses in the LLNA (61). Of the five non-sensitizers, four (zinc sulfate, lead acetate, manganese chloride, and aluminium chloride) failed to induce positive LLNA responses, and only one (copper chloride) tested positive (61). Taken together, these data indicate that, in the great majority of instances, the LLNA provides an accurate assessment of the likely skin sensitizing potential of metal salts, a view endorsed by an independent review in the United States. (81). The argument is, however, rather academic, given that new metals (and therefore metal allergens) are unlikely to be discovered.

The other issue is the possibility of false positive results. One anomaly in the performance of the LLNA is the fact that sodium lauryl sulfate, a non-sensitizing skin irritant, has been shown by some investigators to elicit positive, albeit weak, responses (54,20,50,82). It is possible that Sodium Lauryl Sulphate (SLS) may represent something of a special case, insofar as it is known that this chemical is able to cause the migration of epidermal LC to the skin draining lymph nodes (33), although the relevance of this for the initiation of LNC proliferative activity is not clear. Even if certain skin irritants are able, in some instances, to provoke a comparatively low level of activity in the draining lymph nodes, this does not necessarily compromise the correct interpretation of test data, or prevent the accurate identification of chemicals that have the potential to cause skin sensitization (83). Moreover, it is important to appreciate that the majority of non-sensitizing skin irritants fail to induce positive responses in the LLNA (83,11,27,47,49).

Notwithstanding the above, the question of potential false positive results in the LLNA has remained an important and sensitive topic. Several authors have reported experiences with substances that they consider to elicit false positive responses in the LLNA (84–88). Based upon a past experience with SLS, quite commonly the capacity of such substances to cause skin irritation is cited with the likely explanation of presumptive false positive results. This has even led to suggestions of how to use irritation data in the LLNA, to avoid the production of false positives (89). However, it is clear that this is not soundly based (11). Having said that, it is clear that some specific types of chemistry not normally associated with significant skin sensitizing activity in humans, have proved to be surprisingly positive in the LLNA (85–87). Such knowledge should be used carefully to guide testing and serve as a reminder that whatever skin sensitization test is performed, the results should always be considered in the light of the weight of all the evidence. No test is perfect (not even the LLNA) (90), and regulatory toxicology attempts to adjust this in the light of new knowledge (e.g., (91)), although sometimes imperfectly (11).

It is worth noting that the question of false positives and false negatives applies equally to all toxicology assays, not just those associated with skin sensitization. For the aspect of toxicology under consideration here, the LLNA has had the most focus applied to it, perhaps because the objective, quantitative endpoint does not lend itself so readily to obfuscation. Nevertheless, it is clear that issues exist equally with the guinea pigs assays (92,93). The situation will no doubt continue as we progress to in vitro assays, in no small part due to the conflict between regulatory hazard-based labeling and the actual risk posed by exposure to skin sensitizing chemicals.

INTERNATIONAL REGULATORY STATUS OF THE LLNA

The adoption of a new test method into the regulatory guidelines represents a substantial challenge, demanding both a general scientific consensus on its suitability, as well as acceptance via the formal processes prescribed for validation. As described above, this latter step was undertaken for the LLNA via ICCVAM. The report of this independent review has been published (70). ICCVAM concluded that the method was fully valid as a stand-alone alternative to, or replacement for, the existing guinea pig tests. As a result, the LLNA was adopted by several federal regulatory agencies in the U.S.A. as an accepted method for skin sensitization testing. In addition, the LLNA has been incorporated into a new Test Guideline (No 429; *Skin Sensitization: Local Lymph Node Assay*) by the Organization for Economic Cooperation and Development (OECD) and this was adopted formally in 2002

(94). In parallel, the European Union (EU) prepared a new test method on the LLNA (B42); the text closely followed that developed by the OECD. As a reflection of these developments, the UK competent authority (ca; Health and Safety Executive; HSE), in 2002, effected a change of policy with effect to skin sensitization. The guidance now provided to notifiers by the HSE indicates that the LLNA will be accepted as part of a notification under the *Notification of New Substances* (NONS) regulations. The statement issued by the HSE also stated that, "*The LLNA provides certain advantages with regard to animal welfare (most particularly refinement but also reduction) and also scientific aspects (such as the objective and quantitative nature of the endpoint measured). The LLNA can also provide information on the relative potency of contact sensitizers, unlike other methods currently available for skin sensitization. Given these significant advantages the U.K. ca now considers that for notification purposes the LLNA is the method of first choice for skin sensitization.*" Since that position was reached in 2002, the HSE has accumulated experience with the use in practice of the LLNA within a regulatory context. A retrospective analysis of the LLNA study reports received since the above statement was released, has been conducted recently (95). One conclusion reached was that contrary to some concerns that the LLNA might prove to be either less sensitive or more sensitive than the guinea pig maximization test, the proportion of new substances notified under NONS and classified as skin sensitizers, was comparable with the previous data, before introduction of the LLNA (95,21). More recently, retrospective analyses, from somewhat different perspectives have been conducted in the EU (Casati et al., 2011) and in the U.S.A. (6)

In the light of experiences accumulated with the regulatory use of the LLNA, including those mentioned above and those published elsewhere (96,97) an updated version of the OECD Test Guideline was published (98).

Before leaving the conduct of standard assays and considering the performance of the LLNA, it is appropriate to acknowledge that some other investigators have proposed modifications to the basic protocol—these vary in their scope and complexity. Some suggested changes are relatively modest and conservative, for instance, the use of an alternative isotope or non-isotopic methods for the measurement of LNC proliferation (99–104), or the consideration of the use of mouse strains other than CBA (105). However, other proposals call for much more substantial changes to the standard protocol (62,106–115). Although some of these approaches may have some merit, a detailed commentary on all tests proposed as LLNA variants and modifications is beyond the scope of this chapter, and they will not be considered in detail here. Neither will we discuss here the merits or otherwise of the conduct of the LLNA in species other than the mouse (116–120). However, it is pertinent to note that following the publication of performance standards for LLNA variants (121), two non-radioactive methods have recently been formally validated and adopted in the updated OECD Test Guidelines (91) and have received ICCVAM recommendation in the U.S.A. (122,123).

Finally, it is important to emphasise here that the LLNA was designed initially, and subsequently developed, as a method for the assessment of skin sensitization hazards of chemicals, rather than of complex mixtures or finished product formulations. It is for this purpose that the assay was evaluated and subsequently validated. Despite this, the assay has been deployed for the evaluation of formulations, for example, pesticides, and appears to show some success, hence, its regulatory acceptance/requirement for this purpose (124,81).

THE LLNA AND ASSESSMENT OF RELATIVE POTENCY

Although accurate identification of a hazard is a required first step in any toxicological evaluation, it does not of itself necessarily inform the risk-assessment process. What is really needed, in concert with an appreciation of the likely conditions of exposure, is information regarding the toxicological potency. With respect to the induction of skin sensitization, the potency should be defined as a function of the amount of chemical that is necessary to induce sensitization in a previously naïve subject. Actually the important metric for skin sensitization is the amount of chemical per unit surface area of skin (e.g., $\mu g/cm^2$) (125). In fact, the most compelling illustration of this in humans derives from volunteer studies conducted by Friedmann and colleagues. They have been able to demonstrate that in most circumstances at least, the acquisition of skin sensitization is critically dependent on the amount of chemical experienced per unit area of skin (126). For some time, a major focus of attention has been on defining how the LLNA can be used to assess the relative sensitizing potency of contact allergens experimentally (10,127,128).

The induction by chemical allergens of proliferative responses in draining lymph nodes provides not only a marker of skin sensitizing activity, but also a quantitative correlate of the extent of sensitization (129,130). It is reasonable, therefore, to speculate that it should be possible to determine the relative potency of chemicals on the basis of the vigor of responses induced in the LLNA. For this purpose an EC3 value is derived from the dose-related activity in the LLNA; an EC3 value is defined as the amount of chemical (absolute amount of chemical or chemical per unit area, or percentage or molar concentration) that is required to induce a threshold positive response in the assay (an SI of 3).

Careful thought was given to the most suitable method for deriving EC3 values from LLNA dose-responses. Investigations were conducted in which three possible approaches were compared: quadratic regression analysis, Richard's model, and simple linear interpolation. The conclusion drawn was that linear interpolation between values on either side of the three-fold SI on an LLNA dose-response curve provided the most robust and most convenient method for calculation of EC3 values (131). This approach could be expressed mathematically as:

$$EC3 = c + [(3–d)/(b–d)] \times (a–c) \qquad (39.1)$$

where (a,b) and (c,d) are the coordinates, respectively, of the data points lying immediately above and immediately below the SI value of 3. Where the data points do not meet the criteria for interpolation, a cautious approach to extrapolation has also been defined (132).

It could be argued that there are more sophisticated approaches available for interrogation of dose-response relationships and that the application of these might provide for greater accuracy (115). Although it might appear scientifically heretical to reject such methods in favor of the much more straightforward approach of

linear extrapolation, as will become apparent, it is neither necessary nor helpful for classification purposes, to measure with great accuracy, small and probably biologically insignificant differences between chemical allergens, in terms of EC3 values.

Experience to date reveals that EC3 values are very robust parameters of LLNA responses, both with time within a single laboratory, and also between laboratories (reviewed in (133). Thus, for instance, it was found that the EC3 values were very consistent, ranging from 6.9% to 9.6%, in the studies of HCA, conducted by a single laboratory, over a 10-month period (40). Similar consistency was found when the EC3 values for *p*-phenylenediamine (PPD) were measured each month, over a four-month period (134). Consistency of derived EC3 values was reported also with sequential analyses of isoeugenol (135). The results of inter-laboratory collaborative trials of the LLNA demonstrated that very similar EC3 values were derived when the same chemical was analyzed in several independent laboratories (48–50,134).

In practice, EC3 values have been used successfully to determine the relative skin sensitizing potency of several series of chemicals, including isothiazolinone biocides (57), dinitrohalobenzenes (136), and various aldehydes (137).

The real test of the utility of relative potency measurements based on EC3 values is the extent to which they are congruent with what can be gleaned concerning intrinsic human potency via assessment of the activity of sensitizing chemicals among human populations. To address this issue, analyses were undertaken in partnership with clinical dermatologists, who provided a view of the relative skin sensitizing potency of a series of known human contact allergens. The chemicals were classified according to their relative potency based on clinical judgment and experience. It is essential to understand that this classification was based on a view of intrinsic potency and not at all on prevalence, the latter being highly dependent on exposure. The classifications were then compared with the EC3 values derived for the same chemicals. In each of three investigations, there was a very close correlation between clinical potency and EC3 values (138,139,56). This information has been collated and slightly extended in Table 39.1, which details some 36 substances spread across five potency categories. Using the LLNA-based classification scheme detailed below, approximately 90% of the chemicals are correctly categorized for their intrinsic human potency by the EC3 value.

Based on these analyses, and other investigations, it is relevant to consider how measurement of relative skin sensitizing potency might be best exploited for the purposes of improved classification and labeling. The importance of this derives from the apparent wide variations in the potency. Thus, it is estimated that contact allergens vary by up to four or five orders of magnitude with respect to their relative skin sensitizing potency. This being the case, there is clearly merit in distinguishing between allergens that vary significantly in activity, for the purposes of risk assessment and risk management. This opportunity has been considered in some detail during the last few years (140–143). However, in regulatory practice, it has only proven possible to accommodate two categories, the stronger and weaker sensitizers, with stronger sensitizers being identified by an EC3 value of ≤2% (144). The current regulatory situation is encapsulated in Figure 39.2.

Given the wide potency spectrum of skinsensitizing chemicals, a more comprehensive classification has been proposed: *Extreme* (EC3 <0.1%); *Strong* (EC3 ≥ to <1); *Moderate* (≥1 to <10), and

TABLE 39.1
Comparison of Human Sensitization Potency Category and LLNA Ec3 Values

Chemical	Human Class[a]	LLNA EC3 (%)
Chlorothalonil	1	0.004
Methylchloroisothiazolinone/ methylisothiazolinone	1	0.05
Diphencyclopropenone	1	0.05
p-Phenylenediamine	1	0.06
Potassium dichromate	1	0.08
2,4-Dinitrochlorobenzene	1	0.08
Glutaraldehyde	2	0.2
Propyl gallate	2	0.3
Formaldehyde	2	0.4
Methyldibromo glutaronitrile	2	0.9
Isoeugenol	2	1.3
Cinnamal	2	3.0
Tetramethylthiuram disulfide	2	6.0
Citral	3	13
Eugenol	3	13
Hydroxycitronellal	3	20
Imidazolidinyl urea	3	24
5-Methyl-2,3-hexanedione	3	26
Ethyleneglycol dimethacrylate	3	35
p-Methylhydrocinnamic aldehyde	3	25
Hexylcinnamal	4	8.0
Benzocaine	4	22
Linalool	4	30
Penicillin G	4	46
Propylene glycol	4	NC
Isopropyl myristate	4	44
Propyl paraben	4	NC
Octanoic acid	5	NC
Sodium lauryl sulfate	5	14
4'-Methoxyacetophenone (acetanisole)	5	NC
Isopropanol	5	NC
Lactic acid	5	NC
Glycerol	5	NC
Hexane	5	NC
Diethylphthalate	5	NC
Tween 80	5	NC

[a]Class 1 = extreme; class 2 = strong; class 3 = moderate; class 4 = weak; class 5 = negative; NC = Not calculable.

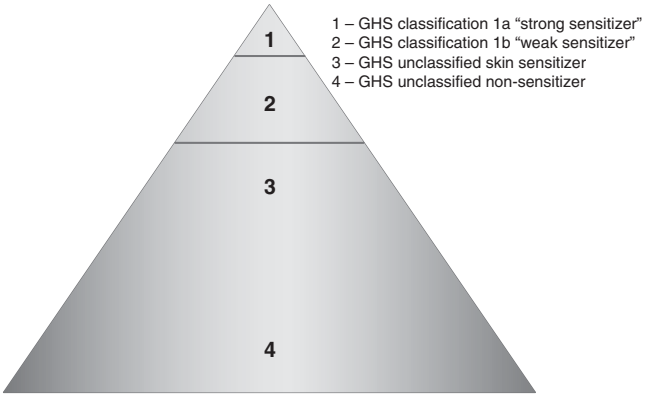

FIGURE 39.2 Skin sensitization potency and regulatory classification.

Weak (≥10 to ≤100) (142). As indicated above, non-sensitizing chemicals would not have a measurable EC3 value, because, by definition, they fail at all test concentrations to provoke a three-fold or greater increase in LNC proliferation compared to vehicle controls. However, it must be emphasized that the above is only one possible classification scheme for grading contact allergens as a function of EC3 values. Nevertheless, it does have the merit of providing a rank order that correlates well with the human experience of sensitizing potential (Table 39.1, adapted from (145)).

Before considering how in practice the relative potency of contact allergens based on EC3 values can be integrated into the risk-assessment process, it is necessary to address one point that was alluded to earlier; the relevance of the vehicle matrix for relative potency. It is clear that the form in which a chemical is encountered on skin surfaces can impact upon the effectiveness with which contact sensitization is acquired, and this is of potential importance in establishing the likely risks to human health. There is good evidence that the vehicle in which a chemical allergen is applied to the skin can have a measurable influence on LLNA responses and EC3 values; the implication being that the vehicle may affect the overall sensitizing potency (reviewed in (10,36)). Perhaps the most significant conclusion to be derived from this study is that although the vehicle does have an effect, it is often no greater than the biological variation associated with EC3 determinations. Only rarely is an effect equivalent to an order of magnitude shift in the EC3 value observed.

It is not possible to draw any general conclusions regarding which vehicles may potentiate skin sensitization. Indeed, such generalizations may not be possible, as experience to date suggests that the impact of vehicle upon the effectiveness of sensitization will vary according to the physicochemical characteristics of the allergen. Notwithstanding these uncertainties, there is every reason to conclude that vehicle formulation can influence the induction of skin sensitization and that it is a necessary consideration when developing risk assessments (see below).

INTEGRATION OF LLNA DATA INTO RISK ASSESSMENT

Skin sensitization risk assessment of new chemicals is a critical step before their introduction into the workplace and/or marketplace. The basic process used for evaluating the skin sensitization risk of a new product ingredient is to consider a no effect level/safety factor approach. This is a stepwise approach that may involve analytical assessments, preclinical skin sensitization testing, clinical evaluation, and benchmarking of the resulting data against similar ingredients or product types (146–148,127,149). It is the potential for an adverse effect to occur in humans exposed, during manufacturing or associated with a product use that is being determined. This approach incorporates assessment of both inherent toxicity and exposure. Specifically, it involves determination of the likely extent of exposure to the test material (exposure assessment), and its sensitization potency (dose-response assessment). It is the ability of the LLNA to assess the skin sensitization potency that makes it an invaluable tool for conducting sound, quantitative, exposure-based risk assessments (150–152).

Despite the importance of potency estimation in the development of accurate risk assessments, there had previously been relatively modest progress in the definition of the appropriate experimental models. The standard guinea pig tests, such as the maximization test, were successful at hazard identification (153,154), and there was some interest in the use of a modified guinea pig maximization test for consideration of relative potency. Of note, was the work of Andersen and co-workers, who manipulated the guinea pig maximization test in order to obtain dose-response data (155).

However, the LLNA has provided a new opportunity for the objective and quantitative estimation of skin sensitization potency (15,127). Specifically, the LLNA EC3 value has been found to correlate not only with subjective assessments of intrinsic human potency (Table 39.1), but also with the threshold data from human experimental models (156,141,143).

These developments led to the formalization of a quantitative risk assessment (QRA) process for skin sensitization. The foundations of this are contained in a pair of publications (158,159). The most detailed exposition of the QRA process is contained in a later publication on fragrance allergens (150). Other worked examples of the QRA have been published for preservatives and transition metals (160,161,14,162).

CONCLUSIONS

The local lymph node assay is now of proven value for the purposes of skin sensitization and hazard identification. It has been formally validated in this respect and has been accepted broadly in the regulatory guidelines, where its objective, quantitative endpoint represents a more attractive option compared with the guinea pig test data. It has also been acknowledged that the assay provides for important animal welfare benefits—fewer animals are needed and animals are subjected to reduced trauma and discomfort. Moreover, it is now acknowledged that, via the EC3 value, the LLNA provides a coherent approach to defining relative potency as an important contribution to the risk-assessment process.

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40 Utilization of irritation data in the local lymph node assay

Peter Ulrich and Hans-Werner Vohr

INTRODUCTION

Lymph node cell proliferation has so far formed the basic paradigm of the local lymph node assay (LLNA) for the identification of contact allergenic potential of low-molecular weight chemicals. The activation of specific effector lymphocytes and the formation of immunologic memory are the hallmarks of a specific immune response with the latter depending on clonal expansion of activated antigen-specific lymphocytes. Consequently, cell proliferation in the lymph nodes (LN) was seen as the indicator of the sensitizing potential of a chemical. However, there is accumulating evidence on irritating chemicals, which cause false-positive results in the LLNA by inducing proliferative events in the skin-draining lymph nodes (1–6). The complex mechanisms leading to irritant-related cell proliferation in the LN are not completely understood, but the phenomenon itself may lead to a reconsideration of the basic principles of the generation and evolution of specific immune responses induced by chemicals with the potential to form hapten–carrier conjugates.

Irritation is often realized as a confounding factor in animal or human testing for contact allergenic potential of low–molecular weight chemicals. Thus, in the biphasic guinea pig tests it is important to treat animals with the lowest still irritating concentration to ensure a successful sensitization while avoiding unnecessary toxicity. Elicitation of contact allergy should be tested with the highest nonirritating concentration to identify a potential lowering of the reaction threshold typical for allergic reactions. In cases of chemicals with a high-intrinsic irritant potential, these testing guidelines may become difficult to follow. Similar hurdles have to be taken in human patch testing, where irritating concentrations of sensitizers may result in false-positive responses. Besides these practical difficulties arising from irritant potential of chemicals, there is a statistical relationship between irritancy and skin sensitizing activity (7). Supporting evidence that the inflammatory response caused by irritation constitutes an important part of the sensitization process, leading to chemical contact allergy, was brought up in attempts to include skin inflammation endpoints into the LLNA (2,4). Either the chemical, its solvent, or an additional chemical in a topical formulation can irritate the skin in a way that proinflammatory cytokines and chemokines are released by epidermal cells. This release forms the initiation of a crosstalk with compartmental immunocompetent cells, such as the Langerhans cells (LCs) in the epidermis, which then migrate to the regionally draining LN where they present antigen to T cells (8). Whether a skin sensitization can happen depends finally on the ability of the chemical to bind to self-structures and form immunogenic hapten–carrier conjugates. The influence of irritation on contact sensitization had been first shown by Magnusson and Kligman (9), who increased the frequency of chemical-sensitized guinea pigs by applying sodium lauryl sulfate to the skin. Grabbe et al. (10) were able to show that sensitized mice could mount a challenge response to a suboptimal dose of the chemical allergen, when a chemically unrelated sensitizer was added at a concentration causing primary irritancy in the skin. Bonneville et al. (11) found that the irritant activity of a hapten increases the severity of contact allergic response. Recently, Jacobs et al. (12) demonstrated in human skin explant cultures that skin irritation by both nonallergenic and allergenic chemicals induced LC migration and maturation underlining that general inflammation induced by irritancy is an important part of the sensitization process. Gene expression analysis in the skin revealed that there is a large overlap between profiles after irritation and contact sensitization and there are only a few genes exclusively responding to sensitizing chemicals (13). In a recent review Kaplan et al. (14) stressed the importance of innate immune recognition of haptens and the related irritant response for the evolution of a contact allergenic reaction. Collectively, published evidence of research in the field of delayed-type hypersensitivity and contact allergy clearly supports the inclusion of irritation endpoints in skin sensitization models in animals.

ASSESSING SKIN IRRITATION WITH LLNA

The first modification of the original LLNA, which utilized incorporation of radioactively labeled thymidine, was the establishment and validation of LN weight and cell counts as endpoints (15). These nonradioactive endpoints provided more flexibility for LLNA by opening it for the use of additional endpoints, such as LN cell phenotyping by flow cytometry and determination of cytokine release (1,16,17). The increasing evidence that pure irritants can cause positive LLNA results, facilitated the introduction of ear thickness or the weight of ear punches to directly relate skin irritation to the LN activation (2,4,18). The establishment of a direct relationship between skin irritation and LN activation in one LLNA study ensures that no additional variables, such as different species, different vehicles, or study designs, which are not comparable to the LLNA may confound data interpretation. There are two methods to assess skin inflammation after chemical irritation in a reliable and easily applicable way: determination of ear thickness with a micrometer and the weight of circular skin samples from the apical area

of the ear using analytic scales. Measuring ear thickness has the advantage of an in-life parameter, and can be used to establish a kinetic of ear swelling during the course of an LLNA study. Care has to be taken that in one study always the same person performs the measurement, because there is a subjective component influencing the handling of the micrometer, the position of the instrument sensors on the ear, and the time when reading the thickness value from the micrometer after placing the instrument sensor on the ear. The last point is crucial, since the fully developed instrument pressure on the ear will squeeze the tissue and thereby causes a continuous decrease in thickness. Ear weights, in contrast, can only be taken once after sacrifice of animals. The advantage, however, is the very low interindividual variation of ear weight data within the vehicle group, increasing the confidence in data from treated groups indicating skin irritation potential of the test chemical. Careful excision of the circular skin samples with a biopsy punch from the apical area of the ear is absolutely necessary. Disregard will lead to larger variation of individual weight data due to the different thickness of the mouse ear from the basis to the apical tip.

Measuring ear thickness or weights can easily be implemented in a routine environment. In an interlaboratory validation of the modified LLNA, ear thickness and ear weight were assessed together with LN weight as well as cell count (19,20). With the exception of two laboratories ear weight data from vehicle-treated groups were within the same range, and all laboratories provided data with a remarkable low intralaboratory variation (Table 40.1). Ear weights were also demonstrated to reflect the differences in irritant potential exerted by the nonsensitizing irritant croton oil and the sensitizer oxazolone while both oxazolone and croton oil induced comparable LN hyperplasia at the applied concentrations (2). In a kinetic LLNA study, it could be shown that croton oil caused a rapid increase in ear weights, whereas oxazalone produced a slower increase and was also less irritating (Fig. 40.1). In a biphasic LLNA using the sensitizer oxazalone (2), the ear weight increase in the challenge group exceeded the changes in the induction control groups indicating that ear weight is a useful marker to demonstrate the increased reactivity in a sensitized animal (Fig. 40.2).

The usefulness of integrating determination of irritant properties of test compounds into the LLNA protocol was also accepted by OECD. The updated OECD guideline TG429 (Skin Sensitization: Local Lymph Node Assay, 2010) includes now the measurement of acute skin reactions by ear thickness on d1 (pre-dose), d3 (approximately 48 hours after the first dose), and d6 (21). Discussions about possible impact of irritation on nonspecific cell proliferation in the draining lymph nodes as well as this update caused several laboratories to check the value of this additional parameter after application of different standard compounds. Some authors claim that measuring ear thickness would not improve the readout of the assay (22). However, this estimation may partially based on the fact that ear thickness was measured exclusively on d6, that is, by ear punch (ear weights) instead of ear swelling on d3 or d4. Acute skin irritation is especially distinct 24 hours after the second (d3) or third application (d4). For this reason measuring ear swelling had originally been incorporated into the protocol on d4 (2,4,18–20). By comparing increases in ear thickness on d4 and d6 in the same study it was found that overall increases due to irritant properties of test compounds were more pronounced and did more often exceed the positive level for this parameter on day 4, but not on day 6. Some examples are given in Table 40.2 (unpublished data by H.-W. Vohr).

The supporting value of the determination of LN hyperplasia and ear irritation in the same LLNA study has also been shown by Gamer et al. (23), who investigated the sensitizing potential of 13 epoxy resins. The endpoints ear thickness and ear weight were

TABLE 40.1
Results of Vehicle Controls (DAE433), Means and Standard Deviations

Laboratory no.	Cell counts (×10⁶ cells/animal)	Lymph node weight (mg)	Ear weight (mg)
1	4.68 ± 1.40	4.93 ± 0.37	22.47 ± 0.31
2	9.71 ± 2.80	5.86 ± 0.60	20.45 ± 1.57
3	7.68 ± 1.47	4.10 ± 0.52	27.00 ± 1.41
4	6.33 ± 1.85	4.10 ± 0.73	19.05 ± 1.02
5	14.69 ± 2.89	6.05 ± 1.05	21.87 ± 1.16
6	9.59 ± 2.27	7.88 ± 1.70	22.40 ± 0.89
7	3.65 ± 0.94	4.13 ± 0.69	22.45 ± 1.08
8	4.60 ± 2.00	5.00 ± 1.00	23.80 ± 1.10
9	10.46 ± 2.13	6.30 ± 0.78	27.52 ± 4.48

Note: Data from an interlaboratory validation.
Source: From Ref. 20.

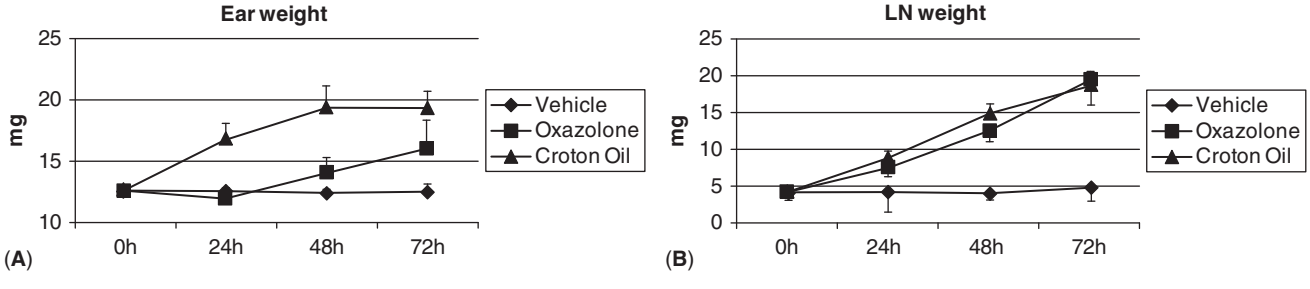

FIGURE 40.1 The kinetics of ear skin irritation and LN activation induced by the contact allergen oxazolone and the irritant croton oil. Six female *Balb/c* strain mice per group were treated on three consecutive days with chemicals on the dorsum of both ears. Twenty-four hours after each application of the test chemical, the respective group of mice was sacrificed and ear weights (**A**) and LN weights (**B**) were determined. Values at "0 hours" refer to untreated animals. Both croton oil and oxazolone were applied in DAE433 at 1% (w/v) and (v/v), respectively. Mean ear weights were computed using individual weights taken from circular pieces (0.28 cm^2) punched from the apical area of one ear. Mean LN weights were derived from pairs of auricular LN per individual animal. *Abbreviation*: LN, lymph node. *Source*: From Ref. 2.

shown to improve the classification of sensitizing chemicals, especially in cases of irritant potential interfering with lymph node endpoints.

RELATIONSHIP BETWEEN IRRITATION AND CONTACT SENSITIZING POTENTIAL

A large set of known standard sensitizers and irritants was tested in a modified LLNA with ear weights and LN weight, as well as cell counts as endpoints (2). A similar approach using ear thickness was reported by Vohr et al. (4). To compare the potencies of the chemicals to induce LN hyperplasia and skin irritation threshold indices were derived from historical data sets. Figure 40.3 plots the concentrations to overcome the threshold index for LN hyperplasia against the respective concentration to overcome the skin irritation threshold index. The plot shows that weak contact sensitizers, such as mercaptobenzothiazole, cinnamic aldehyde, or isoeugenol appeared as weak inducers of LN hyperplasia and showed a weak or no skin irritation potential in the LLNA. The standard irritant sodium dodecylsulfate was located slightly above these weak sensitizers with almost identical threshold concentrations for LN hyperplasia and skin irritation. It is noteworthy that all contact sensitizers with

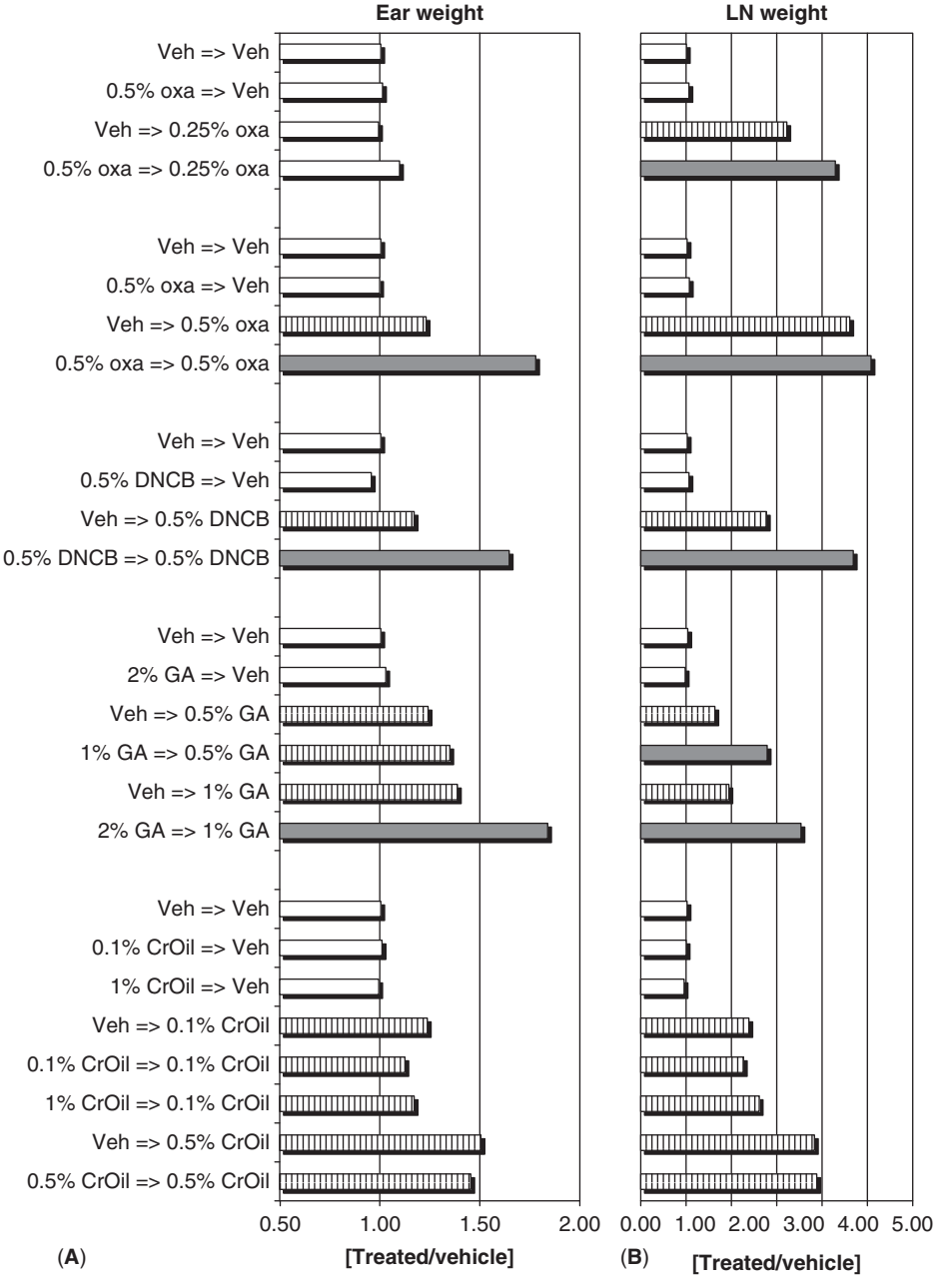

FIGURE 40.2 Challenge responses following secondary exposure to chemicals. Six female *Balb/c* strain mice per group were treated on three consecutive days with chemicals on the shaved back (dorsolumbosacral). Twelve days after the induction phase treatment mice were challenged on the dorsum of both ears for another three days (induction phase => challenge phase treatment). Twenty-four hours following the last exposure ear weights (**A**) and LN weights (**B**) were determined as described for Figure 40.1. Indices were built from treated groups *versus* the vehicle control with an index set to 1. Statistical analysis was performed by comparing chemical-treated groups and the corresponding vehicle controls (striped bars: $P < 1\%$) as well as between challenged groups and the corresponding induction control groups (filled bars, $P < 1\%$). *Abbreviations*: CrOil, croton oil; DNCB, dinitrochlorobenzene; GA, glutaraldehyde; Veh, vehicle. *Source*: From Ref. 2.

a considerable potential to induce LN hyperplasia as oxazalone, dinitrochlorobenzene or dinitrofluorobenzene displayed a marked skin irritation potential. However, also nonsensitizing chemicals, such as the photoirritant methoxypsoralene (8-MOP) and the irritant croton oil appeared in this group of chemicals with a marked potential to induce both LN hyperplasia and skin irritation.

Glutaraldehyde is known to have sensitizing potential, which can be attributed to its capability of covalent binding to various surface proteins. This behavior also represents the reason for its irritation potential, which, at high concentrations, may override clinical manifestation of allergy in the skin. The skin irritation potential of glutaraldehyde in the LLNA determined by ear weight occurs at lower concentrations in comparison to those necessary for the induction of LN hyperplasia (2). Therefore, additional information would be necessary to correctly classify a new chemical in a routine situation with respect to skin sensitization. If there is no evidence from structural considerations that a chemical can cause contact sensitization, or if the primary LLNA gives equivocal result with respect to the specificity, a biphasic LLNA may be conducted. In such a biphasic LLNA, sensitization to glutaraldehyde was achieved with concentrations causing moderate to marked irritation (2). Elicitation of contact allergy in the ear skin and secondary LN hyperplasia was achieved with a combination of 2 and 1% for sensitization and elicitation, respectively. However, significant challenge-related increases in LN weights only, but not in ear weights, were also observed with 1 and 0.5% during sensitization and elicitation, respectively, indicating that the threshold concentration for manifestation of glutaraldehyde-induced ear skin allergy is higher than the threshold for secondary LN activation (Fig. 40.2). In other words, a subclinical manifestation of sensitization at lower concentrations is possible with glutaraldehyde.

Croton oil represents an example on how pharmacologically active chemicals may interfere with the endpoints of the LLNA. Croton oil contains phorbol esters and is a strong inducer of skin irritation and LN hyperplasia. In the lymph node, phorbol esters activate lymphocytes by specific interference in signal transduction via their protein kinase C-activating potential rather than by providing a specific antigenic stimulus (24). Again, ear weight data provide additional information to design a biphasic LLNA to finally clarify the nature of croton oil activity in the LLNA. In such studies no contact allergic potential could be identified at different combinations of sensitization and elicitation concentrations, all of which caused primary changes in skin and LN (Fig. 40.2).

An interesting case highlighting the crucial relationship between skin irritation and sensitization is the cationic surfactant benzalkonium chloride (BC). BC is a known irritant and in rare cases it can be a sensitizer. The diagnosis in human patch testing is often hampered by the marked irritant potential of BC, and thereby increasing the risk of misinterpretation (25). When tested in the modified LLNA, BC produced a bell-shaped concentration–response curve for LN hyperplasia with a peak at 2% and a substantially lower value at 10% (Fig. 40.4). Regarding skin irritation as assessed by ear weights, BC caused a positive concentration–response relationship up to highest tested concentration of 10%. Corroborating results were reported by Woolhiser et al. (26) showing a bell-shaped dose–response curve for LN cell proliferation peaking at 2% in an LLNA and a positive concentration–response relationship up to 5% in a mouse ear swelling test. From these results, it is clear that BC bears a considerable irritant potential, which appears above the concentration of 2% inversely related to LN hyperplasia. The underlying mode of irritant action seems to be different from other sensitizing and nonsensitizing chemicals, which often show a direct correlation between skin inflammation and LN activation. However, there are reports providing evidence for a sensitizing activity of BC in biphasic guinea pig models (25). Maurer (27) was able to elicit a contact allergic response in his guinea pig optimization test either after 0.1% intradermal challenge or after a 10% epicutaneous challenge with 55% positive responses in the first and 21% in the second test setup. The overall conclusion derived from the modified LLNA and the information from other animal tests as well as the human situation is that the foremost activity of BC is that of a skin irritant. In certain, obviously rare cases BC can act as a sensitizer and this may be indicated by the positive LN response at lower concentrations in the LLNA. However, the mechanism behind this inverse reaction pattern in skin and LN remains obscure.

Vohr and Ahr (28) showed that introducing skin irritation assessment could reduce the number of positive LLNA results by resolving unclear cases due to the irritation potential of the test chemical. In comparison to the guinea pig assays (Buehler and Magnusson and Kligman), the incidence of positive LLNA results was then similar over a 2-year testing period investigating up to 74 test chemicals. In a further attempt, evaluation of 120 LLNA studies with different chemicals resulted in 57 (48%) positive results with 29 (24%) cases of skin irritation. From these 29 cases 12 were identified as clear irritants, as verified by a negative secondary response in a biphasic LLNA. Thus, about 20% of all positive results obtained by the determination of cell proliferation in the draining lymph nodes were exclusively due to nonspecific LN cell activation (unpublished data from one of the authors, Vohr).

PHOTOIRRITATION TESTING WITH THE UV-LLNA

In former studies, the suitability of the modified LLNA to test for photosensitizing potential of chemicals had also been demonstrated (1,15). In a further validation of this ultraviolet (UV)-LLNA, the introduction of ear skin irritation data in the UV-LLNA study protocol revealed that assessment of photoirritation provides additional help in determining the nature of the skin reaction—photoallergy or photoirritation (2,4). In addition to epicutaneous administration of chemicals, oral application of test chemicals is also possible and once the chemical is distributed into the skin, the exposure of the mice to a sunlight simulating light source may induce a photosensitization. Chlorpromazine produced different patterns of skin photoreactions and related LN hyperplasia depending on the route of administration. Applied via the oral route chlorpromazine produced significantly more skin photoirritation than LN hyperplasia, whereas after topical application, the LN hyperplasia was more in the foreground (4). A comparable pattern of reactions depending on the route of administration is also observed in humans. The authors concluded that this route-dependent difference in the reaction pattern in the UV-LLNA may reflect a different subcellular distribution pattern of chlorpromazine. After oral application, chlorpromazine tends to distribute more into the nucleus of cells, which results in photon-induced DNA damages and hyperpigmentation of the skin, like with the psoralen 8-MOP. However, after topical application, the partners of photon-induced reactions are more likely proteins on the surface or the cytosol of cells, which then form hapten-carrier conjugates with chlorpromazine leading to contact photoallergy. As a logical consequence one can observe a more pronounced LN hyperplasia after epicutaneous application of chlorpromazine.

TABLE 40.2
Results of Performance Standards as Indicated in OECD 429, SI d4 and d6

Standard compound	Concentrations tested (in %; vehicle)	Cell counts d6 (index)	Ear swelling d4 (index)	Ear Swelling d6 (index)
Sodium dodecylsulfate	1, 5, 10; DMF	1.21, 1.57*, 1.43	0.98, 1.14*, 1.15*	1.00, 1.04, 1.06
Eugenol	2, 10, 50; AOO	1.01, 1.47*, 1,99*	1.04, 1.02, 1.09*	1.02, 1.03, 1.04
Imidazolidinyl urea	2, 10, 50; DMF	1.43, 1.69*, 2.40*	1.02, 0.98, 1.12*	0.98, 0.96, 1.01
Citral	3, 10, 30; AOO	1.37, 2.75*, 3.81*	1.02, 1.02, 1.13*	1.06, 1.04, 1.08
Phenyl benzoate	3, 10, 30; AOO	1.03, 1.47*, 1.33*	1.05, 1.07, 1.14*	1.04, 1.05, 1.05

Abbreviations: DMF, dimethylformamide; AOO, acetone/olive oil, 4:1; Acute ear skin irritation induced by chemicals as related to different time points of determination. Mice were treated with the chemicals as indicated in the table in compliance with OECD TG429 using three different concentrations of the test compounds and the vehicle (second column). Ear thickness was measured on days 4 and 6 as described before (19,20). Indices for ear swelling and cell counts were calculated compared against vehicle-treated animals, which indices were set to 1.0. Indices of all three concentrations are given in the Table in related order.
Source: Unpublished data from H.-W. Vohr; significant data marked by"*".

To clarify whether a chemical is a photoallergen, the assessment of ear weights in biphasic UV-LLNAs becomes an important control in addition to the evaluation of skin reactions as described earlier for contact allergens. Local epicutaneous application of highly lipophilic chemicals leads to rapid systemic distribution, which can last for several weeks (1,2). It was reported that more than two weeks after topical administration of 8-MOP on the shaved back of mice, elicitation of a photoirritation of the ears was possible simply by exposing animals to UVA light without further exposure to the chemical. It is obvious that such an effect, when not carefully controlled, will confound the comparison of skin reactions in animals challenged with chemical and light exposure with those receiving the treatment the first time. It is therefore strongly recommended to introduce a regression control group in UV-LLNA studies to followup the potential photoreactivity caused by retention of test chemicals after primary treatment.

CONCLUSION

Assessment of skin irritation in contact allergy and photoallergy testing with the LLNA has become an important endpoint, which helps to clarify the nature of the reactions observed in this assay. Since induction phase, tests such as the LLNA can detect both pure inflammation by chemical irritancy and allergy-relevant changes in skin-draining LN, it is important to detect the degree of irritation and establish the relation to the proliferative events in the LN. However, even with the addition of skin irritation

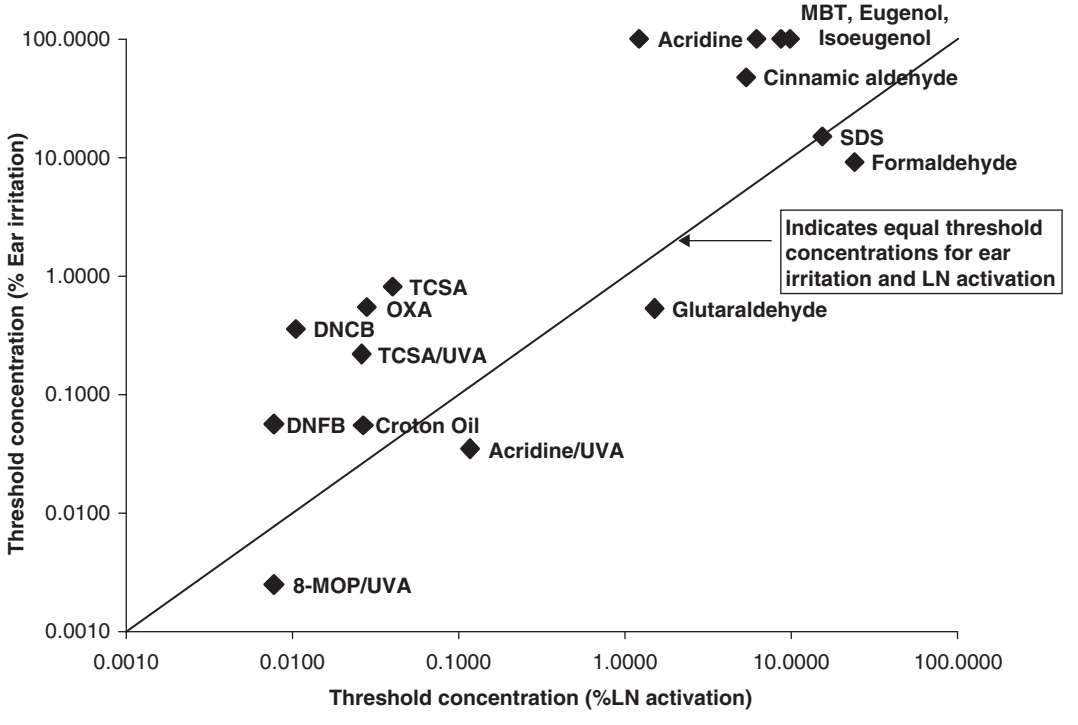

FIGURE 40.3 Relation of primary ear skin irritation and LN activation induced by chemicals. Threshold concentrations for primary LN activation and ear irritation were calculated for each chemical by applying curve fitting algorithms to the concentration–response datapoints. The threshold indices for LN activation (1.3) derived from cell count data and ear irritation (1.1) assessed by weight measurement were approximated from the lowest applied concentrations of the chemicals leading to statistically significant responses. To support the definition of threshold concentrations a large set of historical data was included in the survey. *Abbreviations*: 8-MOP, methoxypsoralene; DNCB, dinitrochlorobenzene; DNFB, dinitrofluorobenzene; GA, glutaraldehyde; LN, lymph node; MBT, mercaptobenzothiazole; OXA, oxazolone; SDS, sodium dodecylsulfate; TCSA, tetrachlorosalicylanilide; LN, lymph node; UVA, ultraviolet-A. *Source*: From Ref. 2.

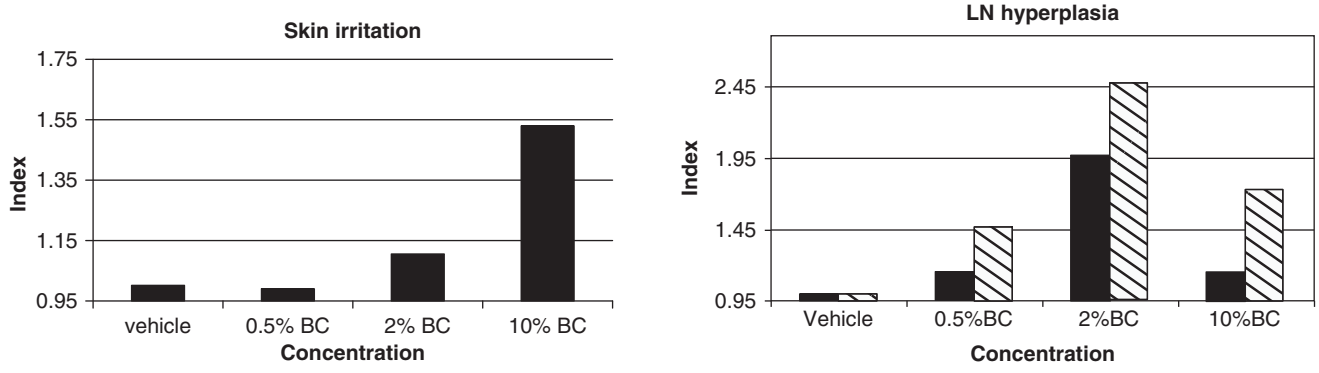

FIGURE 40.4 Concentration–response for BC in the LLNA. Six female *Balb/c* strain mice per group were treated on three consecutive days with chemicals on the dorsum of both ears. Twenty-four hours following the last exposure ear weights and LN weights were determined as described for Figure 40.1. Indices were built from treated groups *versus* the vehicle control with an index set to 1. Filled bars in (**B**) represent LN weight indices, whereas striped bars show LN cell count indices. *Abbreviations*: BC, benzalkonium chloride; LN, lymph node; LLNA, local lymph node assay. *Source*: Unpublished data from Ulrich.

assessment as an endpoint in the LLNA, it is still necessary to use information from structure–activity relationship databases to confirm the hypothesis of a putative contact allergenic potential. There is a fundamental consideration that only a positive result from these *in silico* approaches can be used, since a negative result needs to be proofed by an appropriate *in vivo* model. In cases of uncertainty or lack of a structural alert, it is recommended to conduct a biphasic LLNA to finally clarify the mode of action in mice contact allergy or irritation. Both reactions, when investigated in the early phase, share many features on the histologic level (29). It is also evident that irritants activate a cascade of events with large similarities to those observed during sensitization to contact allergens without leading to a specific activation of T cells (29). Collectively, skin irritation assessment by using reproducible endpoints should be routinely incorporated in every LLNA. They can help with the interpretation of the results of the LLNA, but they cannot overcome completely some natural limitations of induction phase tests.

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41 Failure of standard test batteries for the detection of genotoxic activity of some carcinogenic chemicals used in dermatologic and cosmetic products

Giovanni Brambilla and Antonietta Martelli

INTRODUCTION

Guidelines for the assessment of the genotoxic potential of chemicals are based on the premise that DNA damage and its fixation in the form of gene mutation and chromosomal damage is generally considered to be essential in the multistep process of carcinogenesis, even if genetic damage may play only a part in this complex process. The current guidelines for genotoxicity testing of pharmaceuticals (1–3) indicate a standard three-test battery that consists of (*i*) a test for gene mutation in bacteria, (*ii*) an in vitro test with cytogenetic evaluation of chromosomal damage with mammalian cells or an in vitro mammalian cells gene mutation assay, (*iii*) an in vivo test for chromosomal damage using rodent hematopoietic cells. The recommended genotoxicity tests for cosmetic ingredients (4) consist of (*i*) a bacterial test for gene mutation, (*ii*) an in vitro test for clastogenicity and aneuploidy (metaphase analysis or micronucleus test), and (*iii*) an in vitro mammalian cell mutation assay (mouse lymphoma assay as the preferred choice): further in vivo testing may be justified when concern is raised over positive results in in vitro tests.

In the last years (5), the performance of a battery of three of the most commonly used in vitro genotoxicity tests—Ames + mouse lymphoma assay + in vitro micronucleus or chromosomal aberrations test—has been evaluated for its ability to discriminate rodent carcinogens and noncarcinogens from a large database of more than 700 chemicals. Of the 533 carcinogens with valid genotoxicity data, 93% gave positive results in at least one of the three tests; only 19 carcinogens, out of 206 tested in all three tests, gave consistently negative results in the full three-test battery. On the basis of this evaluation, the European Scientific Committee for Cosmetics and Non-Food Products (ESCCNFP) has reviewed the guidelines for testing hair dyes for genotoxicity (6). This battery of six in vitro tests—bacterial reverse mutation (Ames) test, in vitro mammalian chromosome aberration test, in vitro mammalian cell mutation test, DNA damage and repair (UDS) test in mammalian cells in vitro, in vitro mammalian micronucleus test, in vitro Syrian Hamster Embryo (SHE) cell transformation test—differs substantially from the batteries of two or three in vitro tests recommended in other guidelines. After evaluation of the types of chemicals used in hair dyes and comparison with other guidelines for testing a wide range of chemicals, the ESCCNPF concluded that the potential genotoxic activity of hair dyes may effectively be determined by the application of the three in vitro tests recommended for the genotoxicity testing of other cosmetic ingredients (4); that is, by the same battery of three of the most commonly used in vitro genotoxicity tests judged by Kirkland et al. (5) as a useful tool to identify chemicals possessing carcinogenic or noncarcinogenic potential.

Recently we have put in evidence (7) that there are some chemicals, classified by the International Agency for Research on Cancer (IARC) as probably or possibly carcinogenic to humans on the basis of a sufficient evidence for carcinogenicity in experimental animals, which gave consistent negative results in the test battery for the genotoxicity testing of pharmaceuticals, and in contrast provided positive results in other nonroutinely employed genotoxicity assays. Therefore, it cannot be taken for granted that with this three-test battery the risk of false-negative results for compounds with genotoxic/carcinogenic potential is completely avoided.

The aim of this review is to verify to what extent a series of chemicals used in dermatologic preparations or in cosmetics, which have been found to be carcinogenic in at least one sex of mice or rats, are identified by the three-test (in vitro and in vivo) battery for genotoxicity testing of pharmaceuticals (1–3), and by the three-test (in vitro) battery for genotoxicity testing of hair dyes and of the other ingredients of cosmetics (4).

EXAMPLES OF DRUGS USED IN DERMATOLOGY THAT TESTED POSITIVE FOR CARCINOGENICITY AND GAVE NEGATIVE OR DISCORDANT RESULTS FOR GENOTOXICITY

The Physicians' Desk Reference (8) publishes for several drugs the results of short-term genotoxicity/mutagenicity/clastogenicity assays and of long-term carcinogenicity assays available. Table 41.1 lists, for each of the drugs contained in dermatologic preparations considered in this review, the results of the following assays: long-term carcinogenicity assays in mice and rats; in vitro forward and reverse mutation in *Salmonella typhimurium* and other bacteria; in vitro gene mutation (GM), sister chromatid exchanges (SCE), and chromosomal aberrations (CA) in animal and human cells; in vivo SCE, CA, and micronucleus (MN) formation in hematopoietic

TABLE 41.1
Information Provided by the Standard Three-Test Battery on the Genotoxicity of Some Drugs of Dermatological Use Which are Carcinogenic in Rodents

	Carcinogenicity in rodents			Mammalian cells					
				In Vitro			In Vivo		
Drug	Mouse	Rat	Gene mutation in bacteria	GM	SCE	CA	SCE	CA	MN
Adapalene	nd	+	−	−	nd	−	nd	nd	−
Clorophene	+	?	−	nd	nd	nd	nd	nd	nd
Fluconazole	nd	+	−	−	nd	−	nd	−	nd
Formaldehyde	+	+	+	+	nd	+	nd	nd	−
Griseofulvin	+	+	−	−	−	nd	+	+	−
Hydrogen peroxide	+	nd	+	+	+	+	nd	+	nd
Imiquimod	+	nd	−	−	nd	−	nd	−	nd
Isotretinoin	nd	+	(+)	nd	−	−	nd	nd	−
Mepiramine	−	+	−	+	nd	nd	nd	nd	nd
Pimexcrolimus	+	+	−	−	nd	−	nd	nd	−
Tacrolimus	+	nd	−	−	nd	nd	nd	−	nd
Terbinafine	nd	+	−	−	−	−	nd	−	−
Tretinoin	+	nd	−	nd	nd	nd	nd	nd	−

Note: +, (+), in the presence of at least one positive or weakly positive result in carcinogenicity assays, as well as in genotoxicity assays; −, in the presence of only negative results.
Abbreviations: nd, not determined; GM, gene mutation; SCE, sister chromatid exchange; CA, chromosomal aberrations; MN, micronucleus test.

rodent and human cells. Each drug is then considered separately providing details on the carcinogenesis and genotoxicity assays performed. *Adapalene* (8), which binds to nuclear retinoid receptors and is indicated for the topical treatment of acne vulgaris, when administered in rats by the oral route in doses of 0.15–0.5–1.5 mg/kg/day, that is, up to six times in terms of mg/kg/day of the topical maximum recommended human dose (MRHD), was found to increase the incidence of thyroid follicular-cell adenomas and carcinomas in females, and of benign and malignant pheochromocytomas in the adrenal medulla of males. In a series of in vitro and in vivo assays—Ames test, mouse lymphoma assay, Chinese hamster ovary cell chromosomal aberration assay, and mouse micronucleus test—adapalene did not exhibit genotoxic effects.

Clorophene (9), a chlorinated phenolic antiseptic, stated to be active against a wide range of bacteria, fungi, protozoa, and viruses, is used as skin disinfectant. It was found to increase the incidence of kidney tumors in B6C3F1 male mice treated with 480 mg/kg/day but gave inconclusive results in F344 female rats. Concerning genotoxicity assays, the only available result is the absence of gene mutation in four strains of *S. typhimurium*.

Fluconazole (8), a synthetic triazole antifungal agent, used topically in the treatment of mycosis, was found to increase the incidence of hepatocellular adenomas in male rats treated with 5 and 10 mg/kg/day (i.e., up to seven times the MRHD). With or without metabolic activation, fluconazole was negative in tests for mutagenicity in four strains of *S. typhimurium* and in the mouse lymphoma assay; no evidence of chromosomal aberrations was obtained in vitro in human lymphocytes exposed to 1 mg/mL of fluconazole, and in vivo in bone marrow cells of mice treated by the oral route.

Formaldehyde (10) is a bactericidal disinfectant, also effective against fungi and many viruses, that hardens the epidermis and produces a local anesthetic effect. It was found to increase the incidence of tumors of the nasal cavity in B6C3F1 mice and F344 rats exposed to its vapor, and of leukemia and tumors of the gastrointestinal tract after administration in the drinking water. With or without metabolic activation, formaldehyde gave positive results in six strains of *S. typhimurium* and four strains of *Escherichia coli*, and was found to induce gene mutation, and chromosomal aberrations in in vitro assays, but in vivo tested negative for micronucleus formation.

Griseofulvin (11) is an antifungal substance, produced by the growth of *Penicillium griseofulvum*, which is given topically for infections that involve the skin. When administered in diet, it was found to induce the development of hepatomas in Charles River and in Swiss mice, and of thyroid tumors in Wistar rats. With or without metabolic activation it gave negative results in 12 strains of *S. typhimurium* and for the in vitro induction of gene mutation and SCE in mammalian cells; in vivo it tested positive for the induction of SCE and chromosomal aberrations but tested negative for micronucleus formation.

Hydrogen peroxide (12), used as a 3% solution on the skin as cleansing and topical antiseptic agent, when administered in drinking water was found to increase the incidence of duodenal tumors in various strains of mice. With or without metabolic activation, it gave positive results for gene mutation in 15 strains of bacteria, for the in vitro induction of gene mutation, SCE, and chromosomal aberrations in mammalian cells, and for chromosomal aberrations in vivo.

Imiquimod (8), an immune response modifier, indicated for the topical treatment of actinic keratosis, when applied to the backs of mice three times a week for 24 months up to a dose of 5 mg/kg (251 × MRHD) produced a statistically significant increase in the incidence of liver adenomas and carcinomas in males. In contrast, no evidence of mutagenic or clastogenic potential was observed in five in vitro assays (Ames assay, mouse lymphoma assay, Chinese hamster ovary cell chromosomal aberrations assay, human lymphocytes chromosomal aberrations assay, and SHE cell transformation assay), or in three in vivo assays (rat and

hamster bone marrow cytogenetic assay and a mouse dominant lethal test).

Isotretinoin (8), a retinoid indicated for the treatment of severe nodular acne, when given orally in Fischer 344 rats at dosages of 8 or 32 mg/kg/day (i.e., 1.3–4.0 times the clinical dose of 1.0 mg/kg/day), induced a dose-related increased incidence of pheochromocytoma relative to controls. In the Ames test a weakly positive response was noted in *S. typhimurium* strain TA100. In other genotoxicity assays (SCE in human-derived lymphocytes, Chinese hamster chromosomal aberrations, and mouse micronucleus test), the results were negative.

Mepiramine (13), an antihistamine, was found to induce liver tumors in rats. It gave a negative response in both the presence and absence of metabolic activation in six strains of *S. typhimurium*, but tested positive in the gene mutation mouse lymphoma assay in the presence of metabolic activation.

Pimecrolimus (8), a chloro-derivative of the macrolactam ascomycin indicated for the topical treatment of atopic dermatitis, in a 2-year rat dermal carcinogenicity study was found to produce a statistically significant increase in the incidence of follicular cell adenomas of the thyroid in male animals at a dose of 2 mg/kg/day (1.5 × MRHD). In a 13-week mouse dermal carcinogenicity study, lymphoproliferative changes including lymphomas were observed at a dose of 25 mg/kg/day (47 × MRHD). In a mouse oral carcinogenicity study, a statistically significant increase in the incidence of lymphomas was noted in both males and females at a dose of 45 mg/kg/day (258–340 × MRHD). In another rat oral carcinogenicity study, the incidence of benign thymomas was increased in males at a dose of 5 mg/kg/day and in females at a dose of 10 mg/kg/day. A battery of in vitro tests, including the Ames assay, the mouse lymphoma assay, and the chromosomal aberrations assay in V79 Chinese hamster cells, as well as the in vivo mouse micronucleus test, revealed no evidence for mutagenic or clastogenic potential of pimecrolimus.

Tacrolimus (8), used for prophylaxis of organ rejection and in atopic dermatitis, did not increase tumor incidence in mice and rats when administered orally. However, in a 104-week dermal carcinogenicity study performed in mice with tacrolimus ointment (0.03–3%) a statistically significant increase in the incidence of pleomorphic lymphomas was observed in mice of both sexes treated with 0.1% (3.5 mg/kg) ointment, as well as of undifferentiated lymphomas in females treated with the same dose. In contrast, no evidence of genotoxicity was seen in the following assays: *S. typhimurium* and *E. coli* mutagenicity assays, Chinese hamster ovary and Chinese hamster lung mutagenicity assays, in vivo clastogenicity assay in mice, and unscheduled DNA synthesis in rodent hepatocytes.

Terbinafine (8), which is indicated for the topical treatment of onychomycosis, was found to increase the incidence of liver tumors in male rats given a daily dose of 62 mg/kg (2 × MRHD). In contrast, terbinafine did not increase mutations in *S. typhimurium* and *E. coli*, was nonmutagenic in Chinese hamster fibroblasts, and did not increase the frequencies of SCE and chromosomal aberrations in Chinese hamster lung cells. In vivo it gave negative responses in Chinese hamsters for chromosomal aberrations and in the mouse micronucleus test.

Tretinoin (8), a retinoid metabolite of vitamin A, indicated for the topical treatment of acne vulgaris, in a 91-week dermal carcinogenicity study in which CD-1 mice were treated with 0.017% and 0.035% formulations, was found to induce cutaneous squamous cell carcinomas and papillomas in the treatment area of some females. A dose-related incidence of liver tumors was observed at the same concentrations in male mice. These doses (0.5–1 mg/kg/day) are 10–20 times the maximum human systemic dose. Moreover, studies in hairless albino mice suggested that concurrent exposure to tretinoin might enhance the tumorigenic potential of carcinogenic levels of UVB and UVA. In contrast, tretinoin was found to give negative responses in the Ames test and in the in vivo mouse micronucleus test.

A further example of the failure of the standard three-test battery in detecting the genotoxic activity of carcinogens is provided by permethrin (8), which was found to be carcinogenic in mice but gave negative responses in a battery of nonspecified in vitro and in vivo genotoxicity assays.

Finally, it is worth noting that a drug found to be noncarcinogenic in rodents can test positive in genotoxicity assays; an example being *acyclovir* (8) found to be noncarcinogenic in lifetime assays in mice and rats, but positive in 5 of 16 in vitro and in vivo genotoxicity assays.

EXAMPLES OF CHEMICALS USED IN COSMETICS THAT TESTED POSITIVE FOR CARCINOGENICITY AND GAVE POSITIVE, NEGATIVE, OR CONTRASTING RESULTS FOR GENOTOXICITY

The *IARC Monographs* on the "Evaluation of Carcinogenic Risk to Humans" published the results of all short-term genotoxicity/mutagenicity/clastogenicity assays and of all long-term carcinogenicity assays available. Table 41.2 lists, for each of the chemicals used in cosmetics considered in this review, the results provided by the *IARC Monographs* and by other sources on the following genotoxicity tests: in vitro forward and reverse mutation in *S. typhimurium* and other bacteria; in vitro gene mutation, SCE, CA, and MN in animal and human cells; in vivo SCE, CA, and MN formation in hematopoietic rodent and human cells. Taking into account that in some cases the same test was performed in more than one study and the results may be discordant presumably because of differences in the protocol for each test, the number of positive, weakly positive, inconclusive, and negative responses are listed. Each compound is then considered to show results obtained in genotoxicity assays, which differ for the end point examined or for the target cells from those listed earlier.

Aluminum oxide (14), an abrasive agent used as adjunct in the treatment of acne or for removal of hard skin, was found to increase the incidence of peritoneal mesothelioma in mice treated two times intraperitoneally with the dose of 10 mg, and of lung tumors in female Wistar rats given 5–10 intratracheal instillations of 6 mg. Concerning genotoxicity assays, it tested positive in vivo in rats for both micronucleus formation and chromosomal aberrations of bone marrow cells.

Benzoyl peroxide (15) has mild keratolytic properties and antimicrobial action, and is used mainly in the treatment of acne and tinea pedis. It was found to induce the development of squamous cell carcinomas in female SEN mice treated twice weekly for 51 weeks with 0.2 mL of a 100 mg/mL solution applied on the skin. It was nonmutagenic in six strains of *S. typhimurium* in both the presence and absence of metabolic activation, and in vitro did not induce chromosomal aberrations and aneuploidy in Chinese hamster lung CHL cells.

Butylated hydroxyanisole (16), present as a preservative in various skin products, was found to produce benign and malignant tumors of the forestomach in rats and hamsters when administered in the diet.

TABLE 41.2
Information Provided by the Standard Three-Test Battery on the Genotoxicity of Some Compounds used in Cosmetics, which are Carcinogenic in Rodents

	Carcinogenicity in Rodents			Mammalian Cells						
				In Vitro				In Vivo		
Drug	Mouse	Rat	Gene Mutation in Bacteria	GM	SCE	CA	MN	SCE	CA	MN
Aluminum oxide	+	+	nd	nd	nd	nd	nd	nd	+	+
Benzoyl peroxide	+	nd	−	nd	nd	−	nd	nd	nd	nd
Butylated hydroxy-anisole	nd	+	−	−	−	+	+	nd	nd	nd
Chlorodifluorometh-ane	nd	+	(+)	−	nd	nd	nd	nd	−	nd
D&C red No. 9	nd	+	(+)	−	−	−	nd	nd	nd	−
p-Dimethylaminoaz-obenzene	+	+	+	−	nd	+	nd	nd	nd	nd
1,4-Dioxane	+	+	−	−	(+)	−	−	nd	nd	+
HC Blue No. 1 (purified)	+	+	−	−	+	−	−	nd	nd	+
Hydroquinone	+	+	+	+	+	+	+	nd	+	+
Kojic acid	+	−	+	nd	+	+	nd	nd	nd	nd
Lead acetate	nd	+	−	nd	−	+	nd	nd	+	−
Phenacetin	+	+	+	?	nd	+	−	(+)	(+)	+
Selenium sulfide	+	+	+	nd	nd	+	nd	nd	nd	nd
Talc	+	+	−	nd	nd	nd	nd	nd	nd	nd
Titanium dioxide	nd	+	−	−	nd	−	−	nd	nd	nd
Trichloroacetic acid	+	−	+	(+)	nd	−	nd	nd	+	+

Note: +, (+), in the presence of at least one positive or weakly positive result in carcinogenicity assays, as well as in genotoxicity assays; −, in the presence of only negative results.
Abbreviations: nd, not determined; GM, gene mutation; SCE, sister chromatid exchange; CA, chromosomal aberrations; MN, micronucleus test.

No data are available to evaluate its carcinogenicity to humans. On the basis of sufficient evidence for carcinogenicity in experimental animals, the IARC classified butylated hydroxyanisole as possibly carcinogenic to humans (Group 2B) (17). Results provided by the in vitro standard three-test battery (16) were all negative; butylated hydroxyanisole was nonmutagenic to *S. typhimurium* in both in vitro and in a host-mediated assay, did not induce 6-thioguanine-resistant mutants in cultured Chinese hamster ovary cells and V79 cells, and did not increase the frequency of SCE or chromosomal aberrations in Chinese hamster CHL cells or in Chinese hamster DON cells, respectively. However, according to Kirkland et al. (5), butylated hydroxyanisole was found to give a positive in vitro response in the micronucleus test and in the chromosomal aberrations test. Sex-linked recessive lethal mutations were not induced in *Drosophila melanogaster*. It is worth noting that butylated hydroxyanisole has been found to be a strong inducer of oxidative DNA damage in the epithelial cells of rat glandular stomach (18).

Chlorodifluromethane (19), until recently used as propellant in hair sprays, was found to increase the incidence of fibrosarcomas and Zymbal-gland tumors in male rats in an inhalation study. No data are available for evaluating its carcinogenicity to humans. On the basis of limited evidence for carcinogenicity in experimental animals, the IARC (17) judges chlorodifluoromethane nonclassifiable regarding its carcinogenicity to humans (Group 3). Results provided by genotoxicity tests were substantially negative (19–21). Chlorodifluoromethane was found to be weakly mutagenic in *S. typhimurium*, but did not induce 6-thioguanine-resistant mutants in Chinese hamster V79 cells and reverse mutations at the *hprt* locus in Chinese hamster ovary cells. Neither did it increase the frequency of chromosomal aberrations in rat and mouse bone marrow cells. Moreover, chlorodifluoromethane did not induce either mutation or gene conversion in *Saccharomyces cerevisiae*, forward mutation in *Saccharomyces pombe*, or DNA repair in transformed human cells. It also gave a negative response in the dominant lethal test in rats and mice, and in a host-mediated assay with microbial cells.

D&C Red No. 9 (22), a grade of CI Pigment Red 53:1 used in temporary hair dye formulations and in some countries as a lipstick colorant, was found to produce splenic sarcomas in male rats, and to increase the incidence of neoplastic liver nodules in animals of each sex. No data are available to evaluate its carcinogenicity to humans. On the basis of limited evidence for carcinogenicity in experimental animals, D&C Red No. 9 was judged by the IARC nonclassifiable regarding its carcinogenicity to humans (Group 3). D&C Red No. 9 was inactive in all the assays of the three-test battery in which it was examined. It was not mutagenic in *S. typhimurium* with the exception of a weakly positive response in the TA98 strain in the presence of a precipitate. D&C Red No. 9 did not induce mutation at the *tk* locus in the mouse lymphoma assays, or SCE or chromosomal aberrations in Chinese hamster ovary cells. After oral administration it did not cause micronucleus formation in rat bone marrow cells. Moreover, it did not induce DNA repair synthesis in primary rat hepatocytes and in the liver of intact rats. However, the genotoxic nature of D&C Red No. 9 has been put in evidence from its incubation with a rat cecal

preparation under anoxic conditions to reduce the azo bond. The presumed major reduction product, 1-amino-2-naphthol, was mutagenic in the *S. typhimurium* TA100 strain (23).

p-Dimethylaminoazobenzene (24), contained in brillantines, was found to produce liver tumors in rats by several routes of administration as well as in newborn mice, and bladder tumors in dogs treated orally. No data are available to evaluate its carcinogenicity to humans. The IARC classified *p*-dimethylaminoazobenzene as possibly carcinogenic to humans (Group 2B) on the basis of sufficient evidence for carcinogenicity in experimental animals. According the IARC (24), *p*-dimethylaminoazobenzene did not induce reverse mutations in *S. typhimurium* TA1538 in the presence of rat liver microsomal systems. However, they were produced in the same strain by a urinary metabolite of rats fed with this chemical. It was not mutagenic in *D. melanogaster*. However, according to Kirkland et al. (5) it was found to test positive in the Ames assay and for clastogenic activity in cultured mammalian cells, but tested negative in the mouse lymphoma assay. In rats the intraperitoneal injection of tritium-labeled *p*-dimethylaminoazobenzene gave rise to DNA adduct in liver and spleen (24).

1,4-Dioxane (25), a common trace component of cosmetic products, such as shampoos and skin conditioners, was found to produce, when administered orally, an increased incidence of hepatocellular adenomas and carcinomas in mice; tumors of the nasal cavity, hepatocellular adenomas and carcinomas, mesotheliomas of peritoneum, subcutaneous fibromas, mammary adenomas, and fibrosarcomas in rats. By i.p. injection it produced lung tumors in mice. Death from cancer was not elevated in a single small prospective study of workers exposed to low concentrations of 1,4-dioxane. The IARC classified 1,4-dioxane as possibly carcinogenic to humans (Group 2B) on the basis of inadequate evidence for carcinogenicity in humans and sufficient evidence in experimental animals. Most tests for genotoxic activity produced negative result; 1,4-dioxane was not mutagenic in *S. typhimurium*, did not induce gene mutation at the *tk* locus in mouse lymphoma L5178Y cells or chromosomal aberrations in Chinese hamster ovary cells in vitro. A positive result was obtained in only one of five assays in mouse bone marrow cells for micronucleus induction. Moreover, 1,4-dioxane did not induce recessive lethal mutations in *D. melanogaster*, nor DNA repair in primary hepatocytes, rat hepatocytes, or in the liver of intact rats, and also did not bind to DNA of rat liver cells.

However, 1,4-dioxane was found to induce DNA fragmentation in primary rat hepatocytes and in the liver of intact rats, SCE in Chinese hamster ovary cells, and transformation of BALB/c 3T3 mouse cells. In a subsequent study of Morita and Hayashi (26), 1,4-dioxane tested negative in the bacterial reverse mutation assay and in the mouse lymphoma assay. It did not induce chromosomal aberrations, SCE, and micronucleus formation in Chinese hamster ovary cells, but was positive in the mouse liver micronucleus test.

HC Blue No. 1 (purified) (22), a semi-permanent hair dye, administered up to 6000 ppm in the diet, was found to produce hepatocellular adenomas and carcinomas in mice of each sex, and to increase the incidence of thyroid follicular cell adenomas in males. An increased incidence of pulmonary adenomas and carcinomas was seen in female but not in male rats. No data are available to evaluate its carcinogenicity to humans. It is classified by the IARC as possibly carcinogenic to humans (Group 2B). Results provided by the standard three-test battery were substantially negative. Purified HC Blue No. 1 was not mutagenic in bacteria, did not bind to DNA of the *S. typhimurium* strain TA98 in vitro; did not induce gene mutation at the *hprt* locus in Chinese hamster lung V79 cells or at the *tk* locus in mouse lymphoma L5178Y cells. It increased SCE frequency in one of two assays in Chinese hamster ovary cells but not the frequency in the same cells of chromosomal aberrations. In vivo, a positive response in the mouse micronucleus test was seen in female ICR mice but not in males of the same strain or in CBA and CD-1 mice. In contrast, it elicited DNA repair synthesis in primary cultures of hepatocytes from mice, rats, hamsters, rabbits, and monkeys, but not in human HeLa cells. It is worth noting that commercial nonpurified HC Blue No. 1 preparations were substantially positive in the standard three-test battery.

Hydroquinone (27), used in the treatment of skin hyperpigmentation, was found to produce the following: hepatocellular adenomas in B6C3F1 female mice, when given by gavage at the dose of 50 mg/kg/day, and in males of the same strain when present at the concentration of 0.8% in diet; renal tubule cells adenomas in Fischer 344/N male rats treated by gavage with 50 mg/kg/day, mononuclear cell leukemia in female of the same strain given by the same route 25 mg/kg/day, and renal tubule cell adenomas in male Fischer 344 rats when present at the 0.8% in diet. In vitro, hydroquinone in the absence of metabolic activation was mutagenic in *S. typhimurium* strains TA102 and TA104 but not in other five strains; induced gene mutation in mouse lymphoma L5178Y cells at the *tk* locus, and in Syrian hamster embryo cells at the *hprt* locus and for ouabain resistance; increased the frequency of SCE in Chinese hamster ovary cells, in Syrian hamster embryo cells, and in human lymphocytes; tested positive in the micronucleus assay in Chinese hamster CL-1, V79, XEM2, and SDI cells, as well as in human lymphocytes; induced chromosomal aberrations in Chinese hamster ovary CHO cells, in Syrian hamster embryo cells, and in human lymphocytes. In vivo, it gave positive results in the micronucleus test in bone marrow cells of four strains of mice, and produced chromosomal aberration in bone marrow cells of two strains of mice. Moreover, it was found to induce DNA damage in human promyelocytic HL60 cells, and to bind to DNA of several cell types.

Kojic acid (28), used in cosmetics as skin whitening or depigmenting agent, was found to induce thyroid follicular cell adenomas in male and female B6C3F1 mice fed with diet containing the 1.5% and the 3% of this chemical, respectively. No data are available on its carcinogenicity to humans. In vitro, it was mutagenic in *S. typhimurium* strains TA98, TA100, and TA1535, and increased the frequency of SCE and chromosomal aberrations in Chinese hamster ovary cells.

Lead acetate (29), contained in temporary hair dyes, was found to produce benign and malignant tumors of the kidney in rats following oral or parenteral administration, and gliomas in rats treated by the oral route. Epidemiologic data are considered inadequate. The IARC (17) classified inorganic lead compounds as possibly carcinogenic to humans (Group 2B) on the basis of sufficient evidence for carcinogenicity in experimental animals. Lead acetate was not mutagenic in bacteria either in vitro or in a host-mediated assay. There are conflicting reports on the effect of lead acetate on chromosomal aberrations in cultured mammalian cells and in rodents, but any increase in the frequency of micronuclei in bone marrow cells was absent. It tested negative in *S. cerevisiae* for mitotic recombination.

Phenacetin (17,30), present in bleaching solutions and in permanent-wave preparations, was found to produce benign and

malignant tumors of the urinary tract in mice and rats, and of the nasal cavity in rats when given orally. Evidence for carcinogenicity in humans is considered limited. The IARC (17) classified phenacetin as probably carcinogenic to humans (Group 2A) on the basis of sufficient evidence for carcinogenicity to animals. According to IARC (17,21,30), phenacetin was not mutagenic to bacteria in several assays and resulted positive only when tested in the presence of a metabolic system-derived hamster liver (not from mouse or rat liver). It was negative in a host-mediated assay. Phenacetin did not induce recessive lethal mutation in *D. melanogaster* or DNA fragmentation in cultured mammalian cells. It produced a positive result in an in vitro chromosomal aberration test in Chinese hamster cells in the presence but not in the absence of metabolic activation. The results of studies on the induction of chromosomal aberrations, SCE, and micronucleus in rodents treated with phenacetin in vivo were contradicting. According to Kirkland et al. (5), phenacetin tested negative for micronucleus formation in vitro, and equivocal in the mouse lymphoma assay. It was found positive (31) for DNA damage in the rat kidney.

Selenium sulfide (17,32) has antifungal and antiseborrheic properties, and is used in the treatment of pityriasis versicolor. It was found to produce lung tumors and hepatocellular carcinomas in B6C3F1 female mice treated by gavage with 20–100 mg/kg, and hepatocellular carcinomas or neoplastic nodules in F344 rats treated by gavage with 3–15 mg/kg. It was mutagenic in *S. typhimurium* strains TA97 and TA100, and gave rise to chromosomal aberration in CHO-LB cells.

Talc (33), being soft to the touch and inert has been valued for centuries as a body powder, and today plays an important role in many cosmetic products. In male and female Fischer 344/N rats exposed by inhalation to an aerosol of 18 mg/m^3 an increased incidence was observed of alveolar/bronchiolar adenomas and carcinomas as well as of pheochromocytomas. Marsh mice given a single intrathoracic injection of 10 mg USP talc developed lung tumors. Talc was not mutagenic in *S. typhimurium* strains TA97, TA98, TA100, TA102, TA1535, TA1537, and TA1538.

Titanium dioxide (34), contained in skin and nasal products, was found to produce an increased incidence of lung adenomas in rats of both sexes, and squamous cell carcinomas in females. The only available epidemiologic study provided inconclusive results. On the basis of limited evidence for carcinogenicity in humans, the IARC judged titanium dioxide nonclassifiable as to its carcinogenicity in humans (Group 3). According to the IARC titanium dioxide was nonmutagenic in *S. typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538, and in *E. coli* WP2 *uvrA*. Moreover, it did not induce cell transformation. According to Kirkland et al. (5), titanium dioxide tested negative not only for bacterial mutagenicity but also in the mouse lymphoma assay, and for chromosomal aberrations and micronucleus formation in cultured mammalian cells.

Trichloroacetic acid (35) is used as a caustic on the skin or mucous membranes to treat local lesions and for the treatment of various dermatologic diseases. On the basis of inadequate evidence for carcinogenicity in humans and limited evidence for carcinogenicity in experimental animals, the IARC (35) judged trichloroacetic acid nonclassifiable as to its carcinogenicity to humans (Group 3). In female B6C3F1 mice given 6.67 mmol/L in drinking water it was found to produce a statistically significant increase in the incidence of hepatocellular carcinomas. It was mutagenic in the *S. typhimurium* strain TA100 but not in the other six strains. Moreover, it was weakly mutagenic in the mouse lymphoma L5178Y cells, and gave positive responses in bone marrow cells of Swiss mice for both micronucleus formation and chromosomal aberrations.

CAUSES OF FALSE-NEGATIVE RESULTS

In vitro and in vivo short-term genotoxicity assays have been developed to enable the detection of genotoxic agents, and a battery of such tests should ensure that a chemical, which could potentially induce cancer in humans, does not escape the preliminary phase of screening. Unfortunately, it has been ascertained, and is now generally recognized, that none of the available short-term tests are capable of detecting all genotoxic chemicals (7,36) It is therefore useful to examine separately the causes of false-negative results in both in vitro and in vivo tests. As indicated previously for genotoxicity testing of pharmaceuticals an in vivo test is also recommended (1–3), whereas the genotoxicity testing of hair dyes and other cosmetic ingredients usually only require in vitro tests (4,6) to be performed.

In Vitro Assays

Most carcinogens require biotransformation to DNA reactive species to exert a genotoxic effect, but in both bacteria and mammalian cells that are routinely employed as targets for in vitro tests the enzyme systems involved in the metabolism of xenobiotics are either lacking or expressed to a limited extent. To circumvent this obstacle, tests are carried out in both the absence and the presence of an exogenous metabolic system—usually the rat liver S9-mix derived from animals pretreated with inducers, such as phenobarbitone, β-naphthoflavone, or Aroclor 1254—but this metabolic system should only be considered a first approximation of what occurs in mammalian cells and in the intact animal (37). Other complicating factors may be the qualitative and quantitative differences in the biotransformation of chemicals in different cell types and in cells from different species (38,39). In assays comprising rat liver S9 preparations only, interspecies differences in the metabolism of xenobiotics should not be overlooked, since the animal species from which the liver S9 is obtained may be determinant for the efficient detection of carcinogens as mutagens (40). Taking into account that the aim of the assays is the assessment of human risk, difference in the metabolism between rodents and humans are of fundamental importance. Comparison of mutagenicity data obtained with human liver preparations with those obtained with rat liver preparations showed great interspecies differences in the capacity to activate certain chemicals (41,42), and a large interindividual diversity in the mutagenic response to mutagens of human S9 fractions (42,43). As an example of fact, the rat and human P450 enzymes can differ in their substrate selectivity and reactions catalyzed; in particular the CYP2 family demonstrates vast differences in metabolism between rats and humans. Other possible causes of a false-negative result include the following: metabolism at high doses may be qualitatively and quantitatively different from that occurring at pharmacologically relevant doses, due to test compound-induced inhibition of the formation of downstream metabolism or competition with metabolites for further metabolism; dimethyl sulfoxide, often used as solvent in in vitro tests, inhibits several P450-mediated reactions even at low concentrations (44); finally, it must be considered that

some carcinogens are activated to DNA-damaging species of so short half-live that can react only with the DNA of the cell in which they are formed (45). It cannot be excluded that this may happen, for example, in epidermal cells.

It is generally accepted that none of the in vitro short-term tests can detect all genotoxic carcinogens. The Ames test has been found to poorly detect carbamyls and thiocarbamyls, phenyls, benzodioxoles, polychlorinated aliphatic, cyclic, and aromatic hydrocarbons, steroids, antimetabolites, and symmetrical hydrazines (46). The 10% of rat carcinogens with structural alerts are nonmutagenic in *S. typhimurium* (47). An analysis of data on the clastogenicity of 951 chemicals revealed that 26 out of 111 sufficient positive carcinogens were evaluated as negative, some of them being genotoxic and with structural alerts (48). The mouse lymphoma assay that is capable of detecting chemicals acting as point mutagens, as well as those causing some types of chromosomal aberrations, evaluated as negative 24 of 107 chemicals classified as sufficiently positive carcinogens (some of which were also in this case genotoxic) (49). Similarly some carcinogens have been found to be nonmutagenic to V79 cells at the *hprt* or the Na+/K+ ATPase locus (50).

In Vivo Assays

In the in vivo test of chromosomal damage in rodents hematopoietic cells, indicated by the guidelines for genotoxicity testing of pharmaceuticals (whereas in genotoxicity testing of cosmetic ingredients it might be justified only by positive results in in vitro tests), the occurrence of a false-negative result may be caused by the pharmacokinetic behavior of the test compound; and a very high dose may inhibit enzyme systems involved in its metabolic activation. Owing to an unequal distribution in the tissues of the body and to differences in the activation/detoxification potential of the various tissues, there are chemicals that induce a significant genotoxic and carcinogenic effect in only one organ or cell type. Evidence of a genotoxic tissue-specific effect has been observed in rats, with some chemicals carcinogenic to the kidney (31,51), the urinary bladder (52), and the thyroid (53), which had previously been found to give contradictory or false-negative results in both the in vitro and in vivo standard batteries of genotoxicity tests. Certainly it cannot be excluded for a substance applied topically to the skin the occurrence of a skin-specific genotoxic effect that would otherwise be undetected in the cytogenetic evaluation of chromosomal damage in bone marrow cells. In fact, the bone marrow hematopoietic cells, which are the target in the in vivo assays indicated in the three-test standard battery for genotoxicity testing of pharmaceuticals, have a low biotransformation capacity, and reactive species of short half-life produced in the liver or other organs may be unable to reach them. Chemicals not easily detected by the bone marrow micronucleus test are aromatic amines, N-nitroso compounds, nitroimidazoles, and haloalkanes (54). According to Morita et al. (55), the mouse erythrocyte micronucleus assay detects only 52% of chemicals classified by the IARC as carcinogenic (Group 1), probably carcinogenic (Group 2A), and possibly carcinogenic (Group 2B) to humans. Finally, it should be considered that the type of response—positive or negative—provided by a short-term in vivo assay may depend on the species, strain, and sex of the animal used, as it was found to occur for carcinogenic activity. Furthermore, an analysis of Purchase (56) revealed that 43 of 250 carcinogens were active in mice alone or in rats alone, and the carcinogenic potency database of Gold et al. (57) demonstrated that many carcinogens are not only species specific, but also strain and sex specific. Taking into account that short-term in vivo assays usually employ mice or rats, and that the phylogenetic difference between humans and these rodents is undoubtedly greater than that between mice and rats, it is evident that extrapolation to humans of results provided by these assays may be subjected to substantial errors.

DISCUSSION AND CONCLUSIONS

As exemplified and discussed in this review, the routinely employed standard test batteries, even if capable of detecting the large majority of genotoxic carcinogens, fails to detect some of them. This limitation has been clearly confirmed by an analysis of results provided by in vitro genotoxicity tests performed in Germany (36). This analysis indicated that 72.2% of chemicals found positive in the bacterial gene mutation assay were negative in both gene mutation and chromosomal aberration assays in mammalian cells, and that more than 80% of the in vitro clastogens were found negative in the bacterial mutation assay. A similar picture is given by our analysis of the results provided by the standard test batteries for some compounds used in dermatology and in cosmetics, which have been found to be carcinogenic in at least one sex of mice or rats. Of the 13 carcinogenic drugs used in dermatology (Table 41.1), eight tested negative in all the genotoxicity assays in which they were examined. For these drugs no clear evidence for a nongenotoxic mechanism exists and no information is available to definitely establish whether these drugs are genotoxic or epigenetic carcinogens. As a matter of fact, the negative results provided by the standard test batteries do not exclude a genotoxic mechanism of action that may be revealed by assays other than those included in these batteries. In contrast, formaldehyde tested positive in the Ames assay, and for gene mutation and chromosomal aberrations in cultured mammalian cells; griseofulvin for SCE and chromosomal aberrations in vivo; hydrogen peroxide in all the genotoxicity assays in which it was examined; isotretinoin for mutagenic activity in bacteria; and mepiramine for gene mutation in cultured mammalian cells.

Concerning the 16 carcinogenic compounds used in cosmetics (Table 41.2), the results provided by the standard test batteries confirm the possibility that these batteries may fail in identifying some genotoxic carcinogens. As a matter of fact, three compounds gave only negative results in the genotoxicity assays in which were examined. However, it should be considered that benzoyl peroxide was tested only for mutagenicity in bacteria and for chromosomal aberrations in cultured mammalian cells, talc only in the Ames test, and titanium dioxide only in the Ames test and for gene mutation, chromosomal aberrations, and micronuclei formation in cultured mammalian cells. The other 13 compounds, even if provided discordant results, gave at least one positive response. Aluminum oxide tested positive for chromosomal aberrations and micronuclei formation, in bone marrow cells of rodents; butylated hydroxyanisole for chromosomal aberrations and micronuclei formation in cultured mammalian cells; chlorodifluoromethane and D&C Red No. 9 for gene mutation in bacteria; *p*-dimethylaminoazobenzene in the Ames test and for chromosomal aberrations in cultured mammalian cells; 1,4-dioxane and HC Blue No. 1 for SCE in cultured mammalian cells and for micronuclei formation in mouse bone marrow cells; hydroquinone in the six assays of the

standard test battery in which was examined; kojic acid in the Ames test, and for SCE and chromosomal aberrations in cultured mammalian cells; lead acetate for chromosomal aberrations in both cultured mammalian cells and in rodents; phenacetin in the Ames test, for chromosomal aberrations in cultured mammalian cells, and for SCE, chromosomal aberrations, and micronuclei formation in rodents; selenium sulfide in the Ames test and for chromosomal aberrations in cultured mammalian cells; trichloroacetic acid in the Ames test, for gene mutation in cultured mammalian cells, and for chromosomal aberrations and micronuclei formation in bone marrow cells of Swiss mice.

It is worth noting that of the 16 compounds used in cosmetics at least seven were identified as genotoxic also by assays not included in the standard batteries, or should be considered genotoxic on the basis of their biotransformation.

Since the standard battery of genotoxicity tests was unable to identify all genotoxic carcinogens, for those that gave negative responses in these batteries, performing other types of genotoxicity assays may be considered a wise decision. This opportunity has been already anticipated by the IPCS harmonization of methods for prediction and quantification of human carcinogenic/mutagenic hazard that stated "minimum criteria testing schemes should not be invoked to prevent the conduct of appropriate, albeit nonroutine assays on chemicals (58). Obviously, it is problematic to establish what chemicals deserve further testing, and how many tests should be performed before a chemical is classified as definitely nongenotoxic. Several tests that could be used for additional investigations of the possible genotoxic activity have been indicated in a previous review (7). They include the detection of covalent DNA adducts, DNA damage, and DNA repair either in various types of cultured mammalian cells from animal and human donors, or in vivo in multiple organs of mice or rats. Concerning those chemicals contained in pharmaceutic preparations used in dermatology as well as in cosmetics, it should be considered that xenobiotic metabolism of the skin differs not only quantitatively but also qualitatively from that of the liver, which is the central organ of xenobiotic metabolism (59). Therefore, reasonable additional testing of their possible genotoxicity may be performed targeting cells of the skin. Such an evaluation may be performed in vitro using one of several skin cell culture models that have been developed, including the three-dimensional reconstructed human skin model (60), as well as testing in vivo the possible genotoxic effect within the skin of animals treated topically with the test compound.

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42 Determination of nickel and chromium allergy, sensitization, and toxicity by cellular in vitro methods*

Monika Lindemann

INTRODUCTION

Nickel sulfate remains the most common contact allergen with a standardized prevalence ranging from 19.7% (central Europe) to 24.4% (southern Europe) (1). The epidemiology of nickel allergy is currently changing in Europe following regulatory intervention on nickel release from consumer products (2). In the United States, the prevalence of nickel allergy is still increasing, which may be explained by the absence of regulations. The frequency of nickel allergy is approximately threefold higher in women than in men (3). Exposure to fashion jewelry is hypothesized as a major cause for this gender-based difference, and an increased susceptibility to contact sensitization in women is discussed controversially (4,5). Following cobalt (II) chloride, dichromate is the third frequent cause for metal allergy, showing variant frequency depending on the exposure [2.4% in the UK (west) *vs*. 4.5–5.9% in the remaining European Union (1). Overall, the prevalence of chromium allergy is still increasing in European countries as well as in the United States (2). Allergy and sensitization correlates with exposure to trivalent or hexavalent chromium, which is used by workers in the tanning, pottery, or metal industry, and by construction workers (6,7). In the case of, for example, construction workers, sensitization is caused by the water-soluble hexavalent chromate, which is one component of cement, a substance with alkaline, abrasive, and irritant properties (8). Professional exposure leads to an increase in the sensitization rate to 20% (9). However, in some Scandinavian countries sensitization rates in construction workers have currently been decreasing due to the addition of ferrous sulfate to cement, which was started more than 20 years ago (10). Since 2005, there has been a similar regulation in the European Union (11).

Whereas nickel sensitization is usually equivalent to nickel allergy, this does not apply for sensitization against chromium. Sensitization, that is, a positive reaction to the patch test, does not always mean allergy, which also comprises specific skin symptoms after environmental exposure. Despite its limited predictive value for metal allergy, patch testing is still the gold standard and it is frequently applied for clinical diagnostics of metal allergy (12). Irritant reactions to the patch test can especially hamper the diagnosis of chromium allergy, for example, when using the standard concentration of 0.5% chromium dichromate as many as one out of two positive reactions are considered to be irritant (13). Furthermore, in chronic cases, allergic and irritant contact dermatitis can hardly be differentiated by clinical symptoms (14). It is commonly assumed that trivalent chromium forms the actual hapten, whereas chromate, containing hexavalent chromium, functions as a prohapten (15). Chromate is most capable of penetrating the epidermal layers, whereas trivalent chromium strongly binds to proteins (16). As tri- and hexavalent chromium ions seem to be involved in sensitization, it appears as reasonable to use both chromium compounds as stimuli for in vitro assays (17).

Apart from the patch test, for research purposes the lymphocyte transformation test (LTT, Fig. 42.1) had been available for a long time to detect nickel-specific (18–29) as well as chromium-specific (17,19,30–33) lymphocyte proliferation. Comparing the LTT with the patch test, an increase in sensitivity of up to 30% was described (34). The LTT is an in vitro assay, which uses peripheral blood mononuclear cells (PBMCs). The cells are cultivated in microtiter plates together with the respective metals. Here, the concentration of metals is essential as high concentrations may be too toxic and low concentrations may be too weak to induce metal-specific cell proliferation (17,28). Usually, after 6–8 days of cell culture lymphocyte proliferation is determined by H3-thymidine (H3-TdR) uptake (17,28). Thus, the LTT can determine metal-specific proliferation of white blood cells circulating in peripheral veins. It examines another compartment of the body than the patch test, which has direct access to the skin.

Another in vitro method for the identification of cellular immune reactions is the ELISpot (Fig. 42.2), allowing the measurement of cytokine production on a single cell level (35). Starting in 2002, there are publications on metal-specific ELISpot assays (17,27,28,36–40). The ELISpot appears as more appropriate to detect metal allergy than the enzyme-linked immunosorbent assay (ELISA), which is hampered by the low frequency of metal-specific T cells (40). Like the LTT the ELISpot uses PBMC. However, it does not determine cell proliferation but production of various cytokines, such as interferon (IFN)-γ, interleukin (IL)-2, IL-4, or IL-10 by using pairs of monoclonal cytokine antibodies. In this assay, each cell clone producing a respective cytokine presents as a colored spot.

The diagnosis of metal allergy could not only be relevant for dermatologists, but also for cardiologists, angiologists, or surgeons because allergic reactions against metals are proposed to be a risk factor for in-stent restenosis and reactions to metal skin clips

*Portions of this chapter are derived from Ref. 17,28.

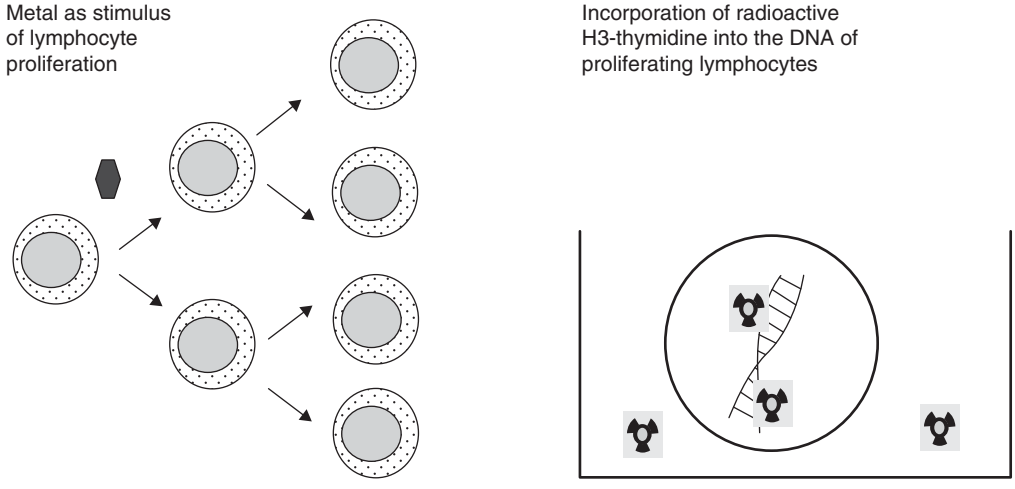

FIGURE 42.1 Schematic depiction of the lymphocyte transformation test.

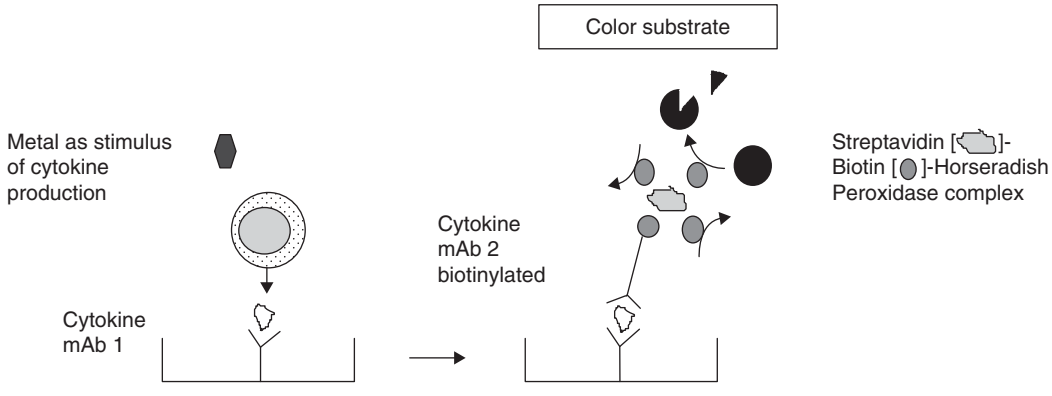

FIGURE 42.2 Schematic depiction of the ELISpot method. *Abbreviation*: mAb, monoclonal antibody.

could result in delayed wound healing (41,42). Of note, apart from allergic reactions, toxic in vivo effects of metals have to be taken into account. For example, in tissues adjacent to stainless steel 316L implants, nickel concentrations ranged between 0.1 and 1.2 µg/mL (43), and in sera from patients with metal implants 1.7 ng/mL nickel concentrations were described (44). While these low concentrations can be quantified by graphite furnace atomic absorption spectrometry, it is currently not defined by a systematic study if they alter cellular in vitro immune responses.

In selected cases, for example, in patients suffering from additional dermatologic diseases (e.g., eczema) or patients with extreme hairiness, in which patch test results are difficult to interpret, a metal-specific LTT and ELISpot—performed by specialized laboratories—could offer an additional diagnostic tool. Compared with the patch test, the LTT and ELISpot are more convenient for longitudinal analysis and do not sensitize patients against test substances. Therefore, it should be a suitable test, especially for the monitoring in clinical studies, for example, on the desensitization/tolerance induction by oral nickel application.

As the diagnosis of metal allergy is still challenging, it was the purpose of our own studies (17,28)—which I will describe in greater detail below—to define the sensitivity and specificity of in vitro tests (LTT and ELISpot) for nickel and chromium allergy. Therefore, patient history and clinical data, patch test, LTT, and ELISpot results were correlated. Apart from the original data, which were published earlier (17,28), this chapter contains new data on nickel toxicity. The analyses presented were only possible due to the close collaboration with experienced dermatologists.

MATERIALS AND METHODS

In altogether 60 volunteers with nickel sensitization, 56 with chromium sensitization, and 45 healthy controls, cellular in vitro methods to detect metal allergy and sensitization were validated.

Study on Nickel Allergy

Study Subjects

In total, 79 patients and medical staff members (65 female; 14 male; mean age, 41 years; range, 14–78) from the Department of Dermatology, Knappschaftskrankenhaus Recklinghausen, were asked for clinical signs of nickel allergy and examined by patch and cellular in vitro tests. Heparinized blood samples were procured after informed consent was obtained. From patch-test–positive and –negative volunteers different numbers of PBMCs and different concentrations of nickel sulfate were studied to define the optimal assay conditions for LTT and ELISpot. In a subset of volunteers, these in vitro assays were performed before and one

week after patch testing to analyze the influence on cellular immunity. Furthermore, kinetic experiments of LTT and ELISpot were performed in parallel to optimize the discrimination between patch-test–positive and –negative volunteers.

Patch Testing

Nickel sulfate (5% in vaseline) was applied by Finn chambers (Epitest Ltd Oy, Tuusula, Finland) to healthy, noninflamed back of the volunteers (45). After 48, 72, and in dubious cases also after 96 hours the reaction was classified according to the guidelines of the German Dermatological Society as negative 0, slightly positive (1+), medium positive (2+), or strongly positive (3+) (46). Positive reactions were defined as the appearance of erythema, papule, and/or vesicle. A crescendo character in patch-test results was considered as typical for allergic, a decrescendo character for toxic-irritable reactions.

Nickel-Specific Lymphocyte Transformation Test

PBMCs from heparinized blood were separated by Ficoll-Hypaque™ density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) centrifugation. The LTT was performed in microtiter plates with 1×10^5 and 2×10^5 PBMCs per 200 µL culture. Seven different concentrations of nickel sulfate (1.6, 3.3, 6.6, 13.1, 19.7, 26.3, and 52.5 µg/mL, that is, 6–200 µM) of nickel (II) sulfate hexahydrate 99%, Aldrich, Steinheim, Germany) were applied. Cell culture conditions and measurement of H3-TdR uptake followed a protocol described previously (47). LTT results were expressed as counts per minute (CPM) increment, that is, lymphocyte transformation with nickel-stimulation minus autologous control (stimulation only with culture medium). For subsequent LTT experiments in parallel to ELISpot kinetics 2×10^5 PBMCs and 13.1 and 19.7 µg/mL nickel sulfate were chosen as conditions yielding optimal results.

Nickel-Specific ELISpot Assay

The ELISpot utilized 3×10^5 and 4×10^5 PBMCs, respectively, and the same nickel concentrations as the LTT. Cell cultures of 200 µL volume were performed in MultiScreen-HA plates (MAHAS4510, Millipore, Bedford, MA, U.S.A.) for 48 hours. Subsequent cytokine production (IL-2 and IFN-γ) was detected by a solid-phase ELISA, using a membrane-anchored first antibody and a biotinylated second antibody at concentrations of 10 and 2 µg/mL, respectively. Clones of antibodies were described in detail previously (28). Using a modified protocol of Herr et al. (48), MultiScreen plates were coated with 60 µL of the first antibody for 2 hours at 37°C in 5% CO_2, followed by three washings with phosphate buffered saline (PBS). Each well was blocked with 150 µL of Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Karlsruhe, Germany), supplemented with 10% of inactivated human serum pool (HSP). After 1 hour at 37°C, the medium was discarded and PBMCs and nickel sulfate were added in RPMI 1640 + 10% HSP. After 48 hours incubation at 37°C the ELISpot plates were washed six times with PBS/0.05% Tween20 (PBS/Tween). Captured cytokines were detected by incubation for 2 hours with 60 µL of the second antibody diluted in PBS/0.5% bovine serum albumin (BSA; fraction V, Calbiochem, La Jolla, CA, U.S.A.) followed by six washings with PBS/Tween. Then, 100 µL of avidin–biotin peroxidase complex (ABC Vectastain-Elite kit, Vector Laboratories, Burlingame, CA, U.S.A.) were added at a dilution of 1:100. ELISpot plates were incubated at room temperature for 1 hour and washed three times with PBS/Tween and three times with PBS. After adding the substrate 3-amino-9-ethyl-carbazole (Sigma, St. Louis, MO, U.S.A.) red spots appeared within 4 minutes. The color reaction was stopped by washing the plates under running water. Numbers of spots were analyzed by the Bioreader 2000 (Biosys, Karben, Germany) and presented as incremental spots.

For kinetic experiments, PBMCs (4×10^5/200 µL culture volume) were preincubated 0, 24, 48, and 168 hours (seven days) with 13.1 and 19.7 µM nickel sulfate in round bottom tubes (TC 163 160, Greiner Bio-One, Frickenhausen, Germany), then 4×10^5 PBMCs per culture were transferred to ELISpot plates and incubated for further 48 hours. To exclude that preincubation induced proliferation of PBMCs before starting the ELISpot, the formation of cell clusters and blast transformation was evaluated microscopically.

Determination of Nickel Toxicity

In eight healthy (nonallergic) controls we performed an LTT using the T-cell mitogen phytohemagglutinin (PHA, 4 µg/mL) or the recall antigen tetanus toxoid (25 µg/mL) as described previously (47). PHA stimulates the majority of T cells and, thereby, proliferative responses to this mitogen determine the "global" T-cell reactivity. In vitro stimulation with the recall antigen tetanus toxoid was chosen because it mimics the in vivo infection with the respective bacterium. Each of the cultures was supplemented with various concentrations of nickel sulfate (between 1.7 ng/mL and 105 µg/mL). Thus, the nickel concentrations reached in the tissue and serum of patients with stainless steel implants (43,44) are covered by this toxicity assay. Each cell culture contained 5×10^4 PBMCs per 200 µL of cell culture medium. Triplicates of PHA-stimulated cells were grown for four days and triplicates of tetanus toxoid-stimulated cells for six days. Cell proliferation was determined by H3-TdR uptake as described (47).

Statistical Analysis

To analyze LTT and ELISpot data at different concentrations of nickel sulfate, the median of seven autologous (unstimulated) values was subtracted from each of the seven nickel-specific values [CPM and spots increment, respectively]. For all other comparisons made, increment values were calculated by subtracting autologous from nickel-stimulated values, considering the second highest of seven values each as described (28). Lymphocyte transformation test results or cytokine production before and after patch testing were compared by Wilcoxon matched pairs test, in volunteers with positive and negative patch-test reactions by Mann–Whitney *U* test. For mutual correlation analysis, Spearman test was applied. Here, results of patch tests were converted into a numeric scale (0, 1+, 2+, 3+ → 0, 1, 2, 3), and medical history was analyzed dichotomously, that is, negative 0 *versus* positive 1. LTT reactions >0 and spots increment >2 for IL-2 and IFN-γ ELISpot were defined as positive. Finally, to assess nickel toxicity, lymphocyte proliferation using various concentrations of nickel sulfate and controls (without this metal) were compared by paired Student's *t* test. Differences were regarded statistically significant at $P < 0.05$.

Study on Chromium Allergy

Study Subjects

In total, 82 volunteers (41 female; 41 male; mean age, 50 years; range, 19–79) also from the Department of Dermatology in

Recklinghausen, answered questionnaires concerning exposure to metals, especially chromium, and clinical signs of allergy. They were all examined by patch and cellular in vitro tests. By patch test, 56 were defined as sensitized and 26 as nonsensitized (healthy controls). In the group of sensitized volunteers 37 reported an anamnesis and symptoms typical (49) of an allergy against chromium (allergic contact dermatitis), whereas 19 did not. The group with *versus* without symptoms of allergy showed a significantly ($P = 0.01$) larger proportion of professional exposure to chromium (59% *vs*. 21%); all other variables included in a questionnaire (see below) as well as total IgE or score of atopy did not differ significantly between the two groups. None of the healthy controls was sensitized against any metal or suffered from skin disease. Following patch testing heparinized blood and serum were collected for cellular in vitro tests and the measurement of total IgE. The interval between patch testing and venipuncture took 7 days to 17 years (median, 63 months). All the volunteers provided written informed consent to participate in the study.

Questionnaire on Exposure to Metals and Clinical Signs of Allergy

The questionnaire recorded the age, gender, time point of a previous patch test, professional or hobby exposure to metals, skin diseases, hyperhidrosis, and clinical signs of allergy. With the help of a medical examination and a face-to-face interview by an experienced dermatologist (possible) symptoms of allergies, which occurred long time ago, were detected, too.

Patch Testing

Potassium dichromate (0.5% in vaseline) was applied by Haye's test chambers (Haye's Service B.V., Alphen aan den Rijn, The Netherlands) to the healthy, noninflamed back of volunteers (49). After 24 or 48 and 72–144 hours, the reactions were classified according to the standard criteria of the International Contact Dermatitis Research Group (50). Patch-test results after 24 and 48 hours occlusion differed as expected; a positive reaction was observed in 25 and 70% of volunteers, respectively, at the first reading. However, the ratio of tests read after 24 and 48 hours did not differ significantly between sensitized volunteers with and without clinical manifestation/symptoms of allergy. A crescendo character in patch-test results was considered as typical for allergic, and a decrescendo character for toxic-irritable reactions. The maximum strength of reactions was classified as weak (1+), moderate (2+), and strong (3+). The maximum strength of patch test reactions was taken for correlation analyses.

Atopy Score

Atopic skin diathesis was recorded based on 24 medical history and clinical criteria according to Diepgen et al. (51), for example, Hertoghe's sign, palmar hyperlinearity, white dermographism, sebostasis, and total IgE. Data were scored and added. Score values of ≥10 were considered as positive.

Chromium-Specific Lymphocyte Transformation Test

PBMCs from heparinized blood were separated by Ficoll-Hypaque™ density gradient centrifugation. 2×10^5 PBMCs in 200 µL culture volume were stimulated with tri- or hexavalent chromium at concentrations of 6, 9, 13, 19, 25, 38, and 50 µg/mL (chromium (III) chloride hexahydrate, 99.995%, Sigma-Aldrich, Milwaukee, WI, USA) or 25, 38, 50, 75, 100, 150, and 200 ng/mL (potassium dichromate >99.5%, crystalline, Roth, Karlsruhe, Germany), respectively, in round bottom microtiter plates (Becton Dickinson Labware, Heidelberg, Germany). Cell cultures were set as sextuplicates. Culture conditions and measurement of H3-TdR uptake followed a protocol described previously (28,47). To optimize the LTT, kinetic experiments were performed for 6, 7, and 8 days. LTT results were expressed as CPM increment.

Chromium-Specific ELISpot Assay

4×10^5 PBMC in 200 µL culture volume were preincubated with tri- or hexavalent chromium in round bottom microtiter plates at concentrations which were also used for the LTT experiments. After 48 hours the cells were transferred to MultiScreen-HA ELISpot plates (MAHAN4550, Millipore, Bedford, MA, U.S.A.). Following further 48 hours incubation in ELISpot plates the resultant spots (representing IFNγ-, IL-2, IL-4, IL-10, and IL-12 producing cell clones) were quantified using an ELISpot plate reader (Bioreader 2000) and results were presented as incremental spots. The respective ELISpot assays were performed as described in detail previously (17,28).

Statistical Analysis

Continuous variables were expressed as mean and standard error of the mean if not otherwise stated. The Mann–Whitney *U* test, Fisher's exact test, or Chi-square test was used to compare different groups as appropriate. We calculated the sensitivity and specificity from the 2×2 contingency tables. The Spearman test was used for correlating variables. GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, U.S.A.) was applied for all calculations. *P* values of <0.05 were considered statistically significant.

RESULTS

Dose Dependency of Nickel-Specific Cellular In Vitro Reactions

In patch-test–positive individuals, nickel-specific proliferative responses showed a clear dose dependency, and concentrations of 13.1 and 19.7 µg/mL of nickel sulfate were found optimal to stimulate 2×10^5 PBMCs in the LTT (Fig. 42.3A). Of note, the highest concentration used in our study (52.5 µg/mL) even suppressed cell proliferation. While IL-2 production to the ELISpot almost mirrored lymphocyte transformation (Figs. 42.3B and 42.4), IFN-γ production showed no clear dose dependency (Fig. 42.3C). As expected, volunteers with positive *versus* negative patch-test results displayed significantly higher ($P < 0.01$) responses to the LTT, the IL-2, and IFN-γ ELISpot (Fig. 42.5).

Influence of Patch Testing on Nickel-Specific Cellular In Vitro Reactions

In order to determine the influence of patch testing on cellular in vitro tests, 23 pairs of analyses (before and one week after patch testing) were performed. It was observed that the in vivo application of nickel sulfate had no significant influence on LTT or ELISpot results. Furthermore, in the total cohort of patch-test positive volunteers there was no influence of the interval between patch testing and venipuncture (mean 2.2 years; range, 1 day to 19 years) on the cellular in vitro parameters.

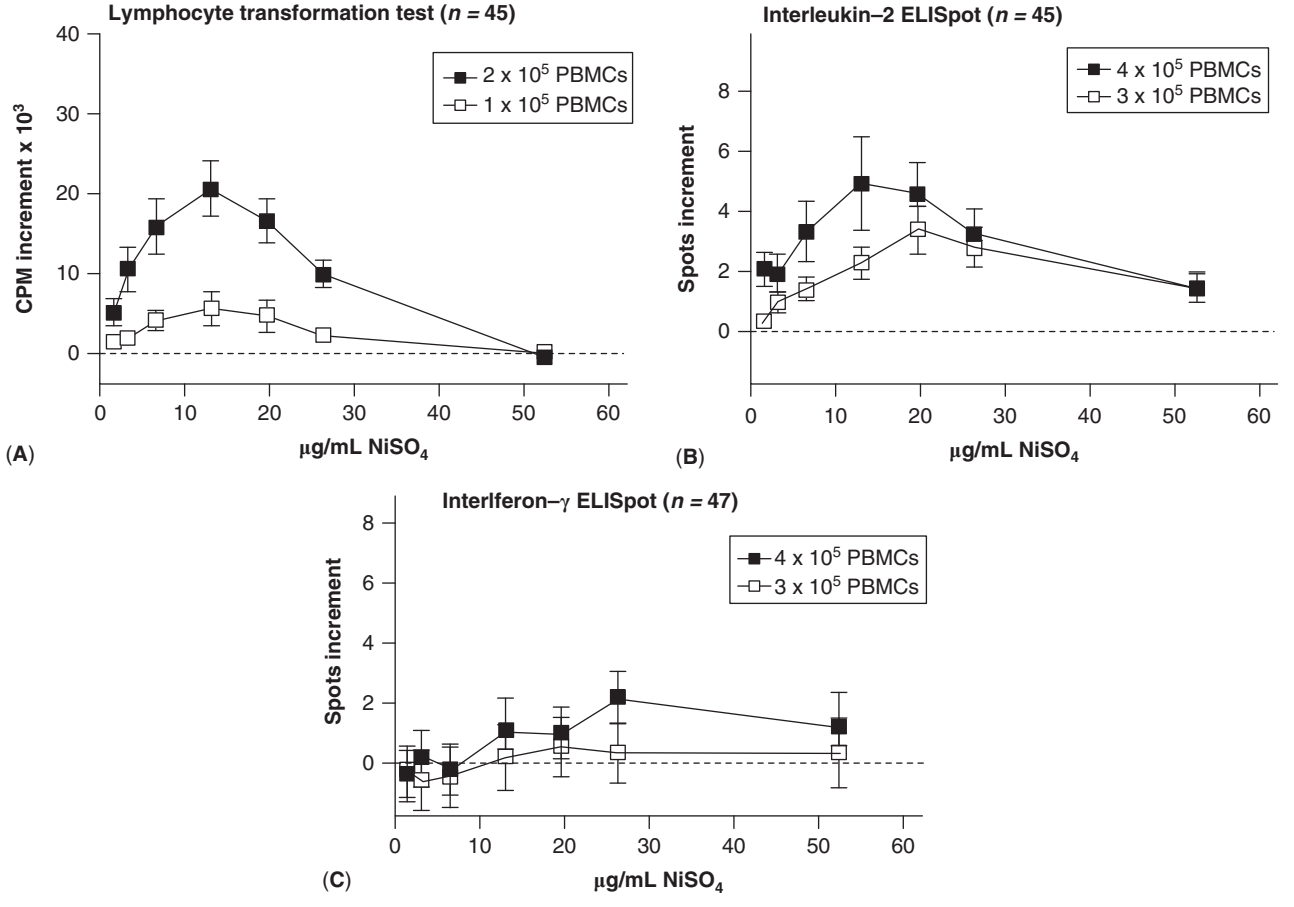

FIGURE 42.3 Lymphocyte transformation test and ELISpot results in patch-test–positive volunteers using different concentrations of $NiSO_4$ and various numbers of PBMCs. Values are given as mean ± standard error of the mean. (Increment means nickel-stimulated minus unstimulated control culture.) *Abbreviations*: $NiSO_4$, nickel sulfate; PBMCs, peripheral blood mononuclear cells; CPM, counts per minute of H3-thymidine uptake.

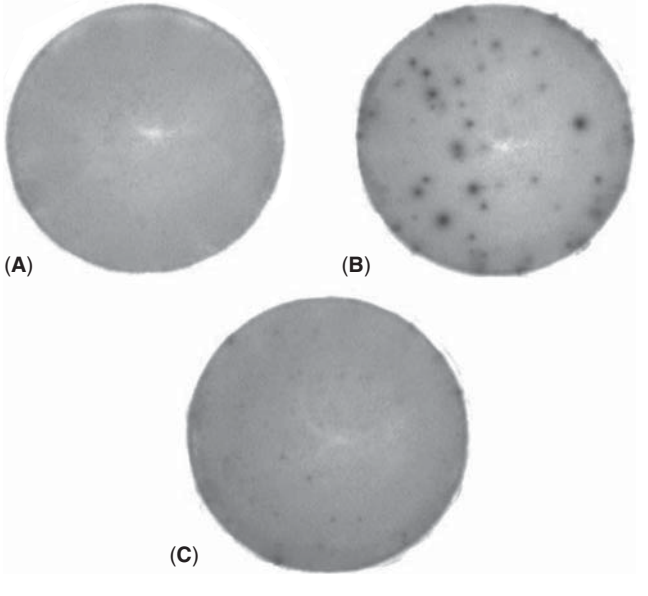

FIGURE 42.4 Interleukin-2 ELISpot results in one nickel-allergic and patch-test–positive volunteer using 3×10^5 peripheral blood mononuclear cells/culture; (**A**) autologous control, (**B**) 13.1, and (**C**) 52.5 µg/mL nickel sulfate.

Determination of the Frequency of Nickel-Specific PBMCs

An increase in ELISpot sensitivity was reached by preincubation ("priming") with nickel sulfate for 24–48 hours prior to transfer to ELISpot plates. In patch-test positive volunteers, an average precursor cell frequency of 1.7×10^5 and 19×10^5 could be defined for IL-2- and IFN-γ-producing PBMCs, respectively. These calculations were based on maximum responses, that is, using a preincubation period of 24 hours for IL-2 and of 48 hours for IFN-γ. In healthy controls IFN-γ–producing cells were also detectable, but with significantly lower frequency (2×10^5, $P = 0.004$). Microscopic analysis revealed that nickel-specific cell clusters and blast transformation started to appear after a preincubation period of 96 hours indicating that the frequency of cytokine-producing lymphocytes following preincubation for 24 and 48 hours was not elevated.

Determination of Nickel Toxicity

Titration experiments with various concentrations of nickel sulfate (between 1.7 ng/mL and 105 µg/mL) indicated that the "global" T-cell reactivity (reaction toward PHA) was not significantly decreased at concentrations reached in the serum of patients with metal implants (1.7 µg/mL) (44) (Fig. 42.6). But at concentrations reached in the tissue adjacent to metal implants (43), a significant ($P < 0.01$) decrease by 12–16% compared with controls could be detected. However, the immune response to more physiologic stimuli, such as tetanus toxoid, may be more relevant for carriers

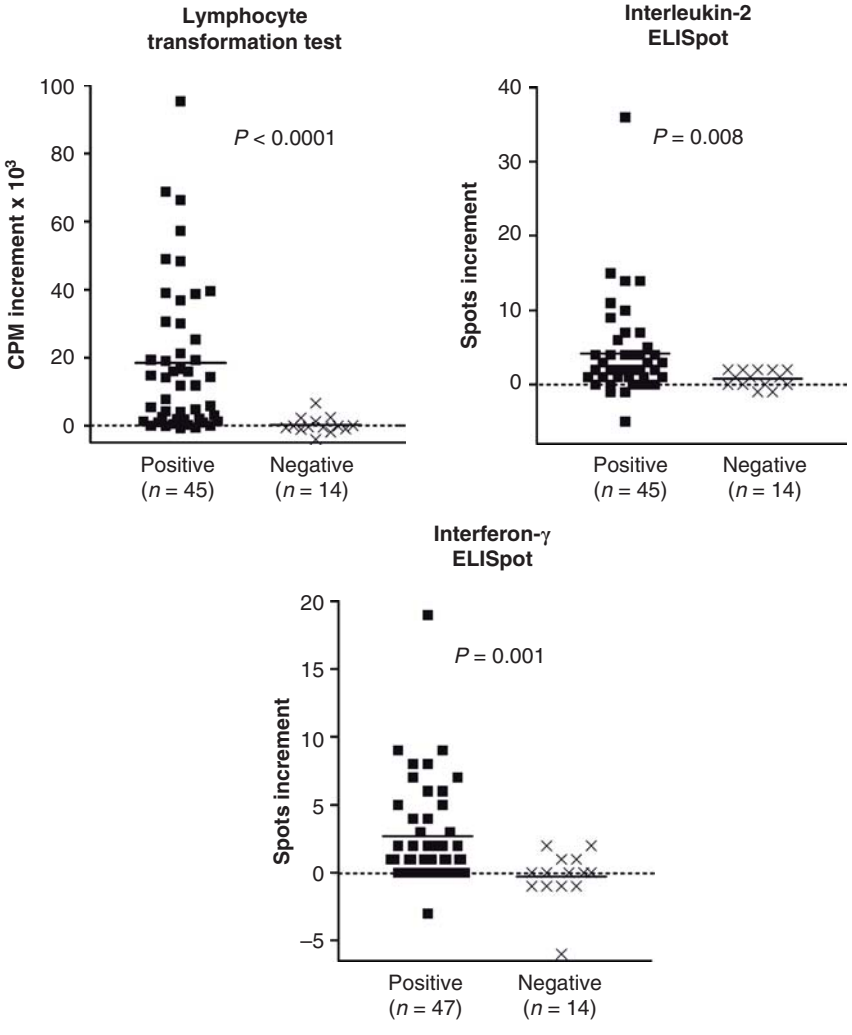

FIGURE 42.5 Lymphocyte transformation test and ELISpot results in volunteers with positive and negative patch-test results and their statistically significant (Mann–Whitney *U* test) differences. For lymphocyte transformation tests 2×10^5 and for ELISpot 4×10^5 peripheral blood mononuclear cells/culture were used. Horizontal lines indicate mean values for the two groups.

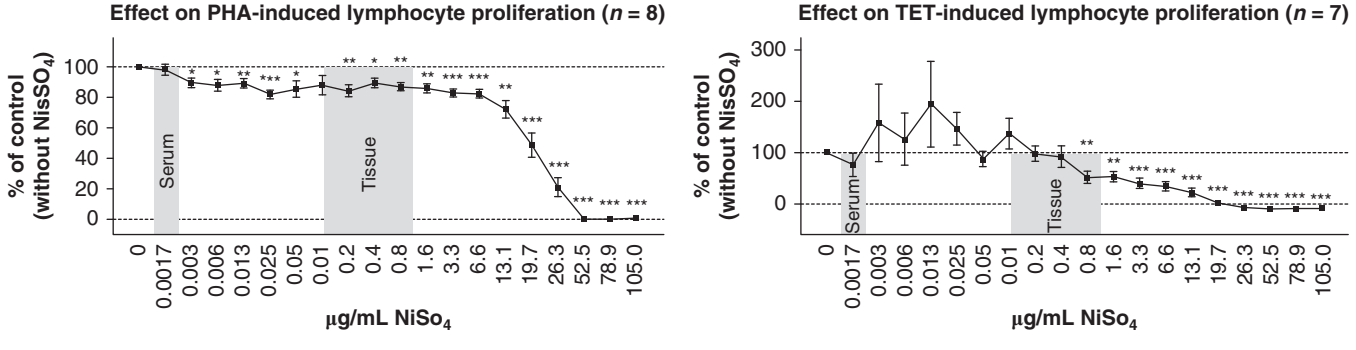

FIGURE 42.6 Effect of $NiSO_4$ (1.7 ng/mL to 105 µg/mL) on PHA- or TET-induced lymphocyte proliferation. Nickel concentrations that can be reached in serum or tissue of patients with stainless steel implants (43,44) are shaded in gray. For lymphocyte transformation tests 5×10^4 peripheral blood mononuclear cells/culture were used. Data are given as mean and standard error of the mean. Lymphocyte proliferation using various concentrations of nickel sulfate and controls (without this metal) were compared by paired Student's *t* test (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). *Abbreviations*: $NiSO_4$, nickel sulfate; PHA, phytohemagglutinin; TET, tetanus toxoid.

of metal implants. Nickel concentrations as reached in the serum did not significantly alter the response toward tetanus toxoid. Remarkably, at the highest concentration that can be reached in the tissue adjacent to nickel-containing implants, the immune response was reduced by 49% compared with controls ($P < 0.01$). Thus, in vivo effects of nickel on antibacterial defense could indeed occur in the tissue adjacent to stainless steel implants.

Correlation of Nickel Allergy, Patch Test, and Cellular In Vitro Test Results

Overall, the parameters nickel allergy, patch test, LTT, and ELISpot results were positively correlated ($P < 0.05$ each; Fig. 42.7 and Table 42.1). Of note, all but one volunteers with nickel allergy were positive to the patch test yielding a sensitivity

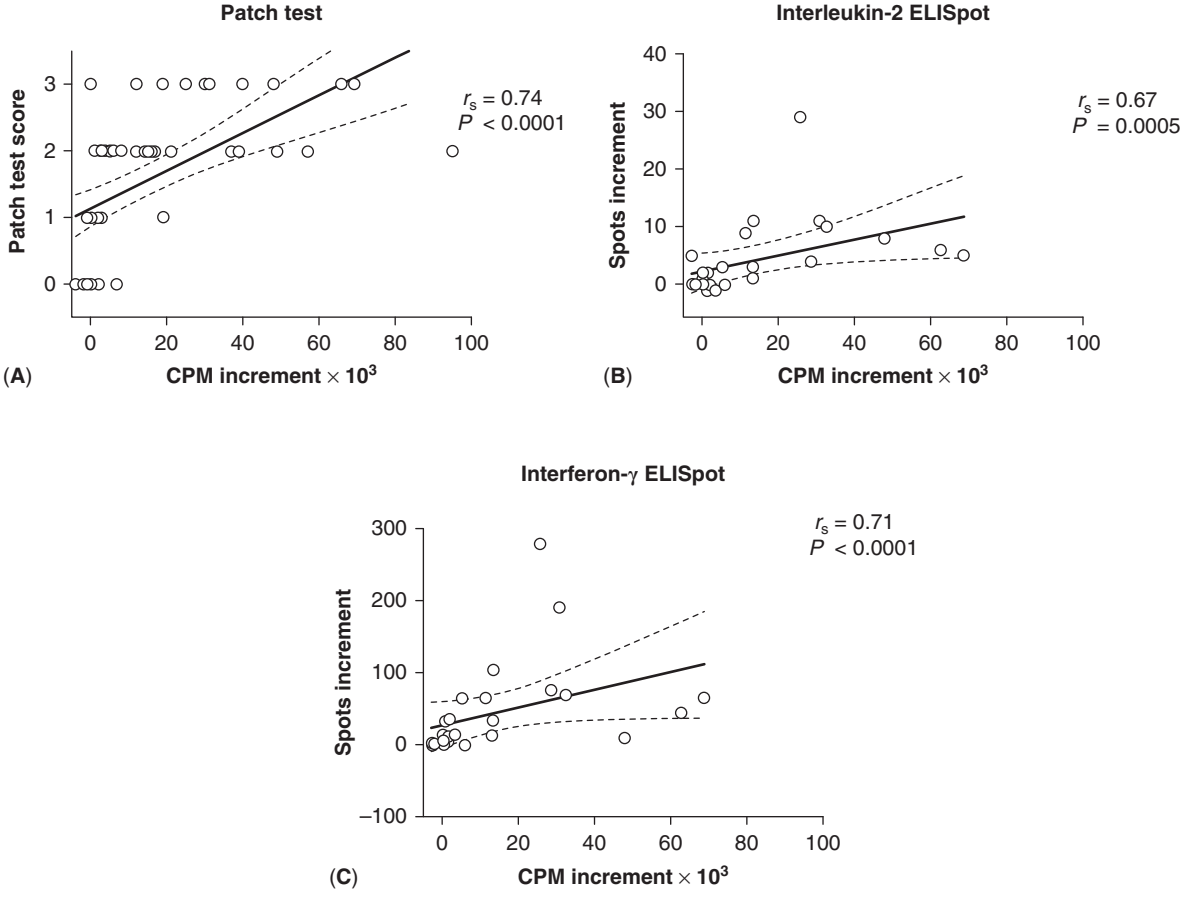

FIGURE 42.7 Spearman correlation analysis between patch test or ELISpot results and lymphocyte transformation test responses. (**A**) Patch-test scores and H3-thymidine uptake ($n = 59$), (**B**) interleukin-2 ELISpot after 24 hours preincubation and H3-thymidine uptake ($n = 23$), and (**C**) interferon-γ ELISpot after 48 hours preincubation and H3-thymidine uptake ($n = 25$).

of 98% (42/43) (Fig. 42.8). However, four patch-test positive individuals did not display nickel allergy. Thus, the specificity of a positive patch test was 91% (43/47). Interestingly, in the four patients with positive patch test but without nickel allergy, the LTT results were significantly lower than in those with nickel allergy (0.1 ± 0.5 *vs*. $21.2 \pm 3.5 \times 10^3$ CPM increment, $P = 0.003$). The same trend holds for IL-2 and IFN-γ ELISpot (0.0 ± 1.8 *vs*. 4.6 ± 1.0 and 0.7 ± 0.7 *vs*. 2.8 ± 0.6 spots increment, respectively). Based on the anamnesis and clinical symptoms of nickel allergy, the LTT was positive in 95% (40/42) of allergic volunteers and in 12% (2/17) of healthy controls. Thus, the sensitivity of the LTT was 95% and the specificity 88%. Concerning the nickel ELISpot, the sensitivity was 87% and the specificity 90%. In addition, LTT and ELISpot results could predict patch test results with a high sensitivity and specificity (approx. 90% each).

Prediction of nickel allergy

Test	Sensitivity	Specificity
Patch test	98%	91%
Lymphocyte transformation test	95%	88%
ELISpot*	87%	90%

*Interleukin-2 and/or interferon-γ ELISpot positive

FIGURE 42.8 Sensitivity and specificity of patch test, lymphocyte transformation test, and ELISpot for the prediction of nickel allergy ($n = 61$).

Proliferative In Vitro Responses in Volunteers with Chromium Sensitization and Allergy

Chromium-sensitized volunteers with allergy ($n = 33$) displayed significantly higher LTT responses than sensitized volunteers without allergy ($n = 16$) and healthy controls ($n = 25$) ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 42.9A). In sensitized volunteers without allergy and controls the chromium compounds appeared as toxic, that is, LTT responses were lower after metal stimulation than in unstimulated cultures. 12.5 μg/mL of chromium chloride and 50 ng/mL of potassium dichromate were found optimal to discriminate between sensitized individuals with and without allergy.

Similar to nickel sulfate, chromium chloride and potassium dichromate induced a dose-dependent LTT response (Fig. 42.10). Here, 7- and 8-day LTT cultures showed greater differences between the three groups than 6-day cultures. Of note, in 7- and 8-day cultures, sensitized volunteers with *versus* without allergy displayed significantly ($P < 0.05$) higher proliferative responses toward both chromium compounds. Furthermore, responses in allergic volunteers were significantly higher ($P < 0.01$) than in healthy controls. Maximum proliferative responses were greater in 7 than in 8-day cell cultures.

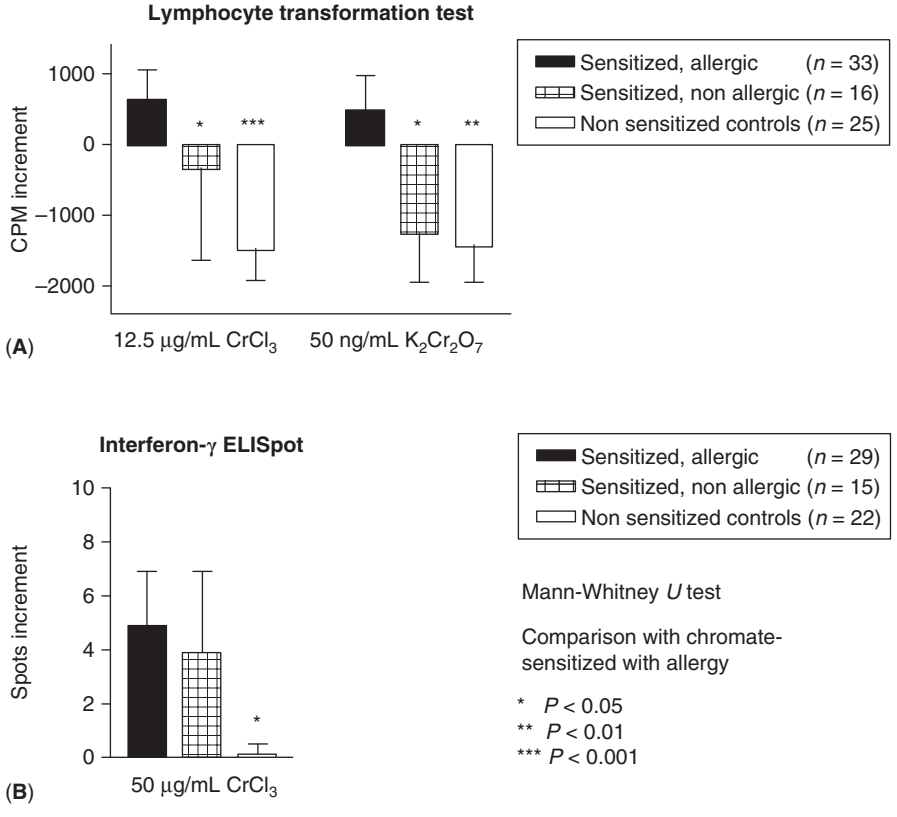

FIGURE 42.9 Lymphocyte transformation test and ELISpot results in volunteers with sensitization against chromium with allergy (black bars), with sensitization without allergy (hatched bars), and nonsensitized healthy controls (white bars). $CrCl_3$ was used at a concentration of 12.5 µg/mL and $K_2Cr_2O_7$ at a concentration of 50 ng/mL in 7-day cultures and 4-day cultures, respectively. Data represent mean and standard error of the mean. (Increment means chromium-stimulated minus unstimulated control culture.) *Abbreviations*: CPM, counts per minute of H3-thymidine uptake; $CrCl_3$, chromium chloride; $K_2Cr_2O_7$, potassium dichromate.

TABLE 42.1
Spearman Correlation Analysis for Medical History, Patch Test, and Cellular In Vitro Parameters

Parameter 1	Parameter 2	r_s	*P* Value
Medical history	LTT	0.70	<0.0001
Medical history	IL-2 ELISpot[a]	0.73	<0.0001
Medical history	IFN-γ ELISpot	0.66	0.0004
Patch test	Medical history	0.89	<0.0001
Patch test	LTT	0.64	0.0006
Patch test	IL-2 ELISpot[a]	0.65	0.001
Patch test	IFN-γ ELISpot	0.67	0.0002
LTT	IL-2 ELISpot[a]	0.67	0.0005
LTT	IFN-γ ELISpot	0.71	<0.0001

In this analysis, 25 ELISpot assays with preincubation [24 hr for IL-2 and 48 hr for IFN-γ], and 25 LTT performed in parallel were considered. For LTT 2 × 105 and for ELISpot 4 × 105 PBMCs/culture were applied.

[a]Two volunteers could not be tested by IL-2 ELISpot. *Abbreviations*: IFN-γ, interferon-γ; IL, interleukin; LTT, lymphocyte transformation test; PBMCs, peripheral blood mononuclear cells.

Using 12.5 µg/mL of chromium chloride as stimulus in 7-day cultures, 18/33 volunteers with sensitization with allergy, 5/16 with sensitization without allergy, and 4/25 nonsensitized healthy controls displayed positive reactions. The respective numbers for stimulation with 50 ng/mL of potassium dichromate were 16/33, 3/16, and 3/25. Combining results of chromium chloride and potassium dichromate LTT, a positive reaction to at least one of the stimuli was observed in 23/33 (70%), 5/16 (31%), and 5/25 (20%) volunteers, respectively. Thus, a positive reaction to either of the chromium compounds is predictive of allergy [sensitization with *vs*. without allergy: odds ratio (OR) = 6.4, $P = 0.004$; sensitization with allergy *vs*. controls: OR = 11.5, $P < 0.0001$]. According to these data, allergy could be predicted with a sensitivity of 23/33 (70%) and a specificity of 31/41 (76%) (Fig. 42.11). Notably, Spearman analysis showed that cellular in vitro responses toward chromium chloride and potassium dichromate were significantly correlated ($r = 0.73$, $P < 0.0001$; 7-day culture, 12.5 µg/mL and 50 ng/mL, respectively).

Chromium-Specific ELISpot Results

On the contrary, the chromium-specific ELISpot assay determining IFN-γ, IL-2, IL-4, IL-10, and IL-12 production was of minor value for the prediction of an allergy against chromium. Volunteers with sensitization with and without allergy did not differ significantly following stimulation with tri- and hexavalent chromium compounds (Figs. 42.9B and 42.12). However, the mean IFN-γ, IL-4, and IL-10 production was slightly higher in volunteers with *versus* without allergy. There was one remarkable finding: Allergic volunteers displayed significantly ($P < 0.05$) higher IFN-γ production following stimulation with chromium chloride (50 ng/mL) than healthy controls (4.5 ± 1.7 *vs*. 1 ± 0.4 spots increment) (Fig. 42.12). Furthermore, LTT and ELISpot results were only weakly correlated.

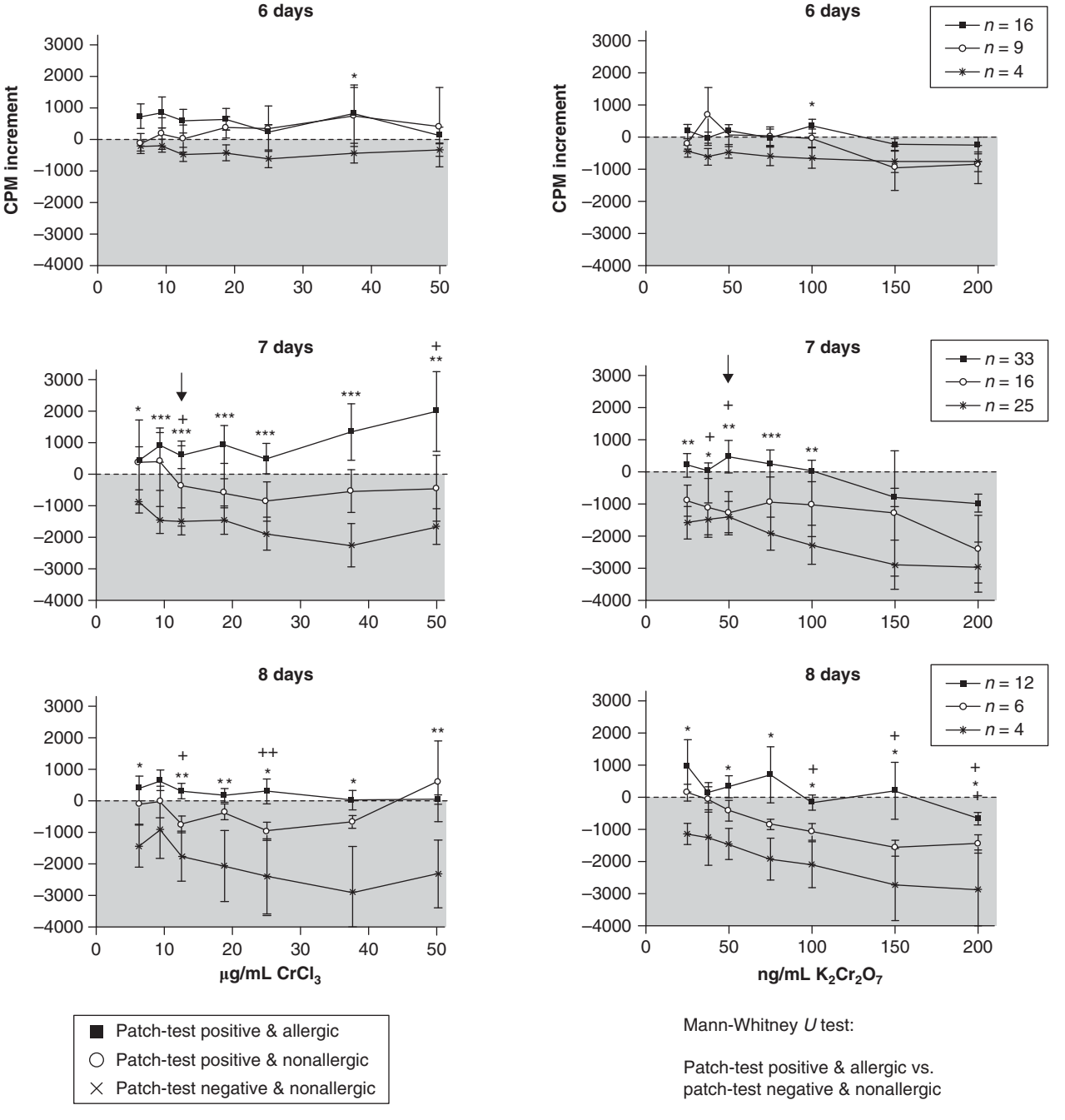

FIGURE 42.10 Dose–response curves of lymphocyte transformation test results using seven different concentrations of $CrCl_3$ and $K_2Cr_2O_7$ in 6-, 7-, and 8-day cell cultures. Data from 37 sensitized volunteers with allergy (filled squares), 19 without allergy (open circles), and 26 nonsensitized healthy controls (asterisk) are given as mean ± standard error of the mean; 4/37, 3/19, and 1/26 volunteers in the respective groups were only tested in a 6- or 8-day culture but not in a 7-day cell culture. Arrows indicate culture conditions found optimal to discriminate between sensitized individuals with and without chromium allergy. Toxic reactions toward chromium compounds are shaded in gray. *Abbreviations*: CPM, counts per minute of H3-thymidine uptake; $CrCl_3$, chromium chloride; $K_2Cr_2O_7$, potassium dichromate.

Prediction of chromium allergy

Test	Sensitivity	Specificity
Patch test	Not defined	61%
Lymphocyte transformation test	70%	76%
ELISpot	Not suitable	

FIGURE 42.11 Sensitivity and specificity of patch test, lymphocyte transformation test, and ELISpot for the prediction of chromium allergy ($n = 74$).

Determination of the Frequency of Chromium-Specific PBMCs

Based on maximum IFN-γ production, an average precursor cell frequency reactive to chromium chloride can be defined as 26, 15, and 11 per 10^6 PBMCs in volunteers with sensitization with allergy, with sensitization without allergy, and healthy controls, respectively (Fig. 42.12). Thus, chromium-specific precursor cell frequency is approximately 14-fold lower than nickel-specific precursor cell frequency (26×10^6 *vs*. 19×10^5).

Correlation of Chromium Allergy, Atopy Score, Patch Test, and Cellular In Vitro Test Results

Most importantly, clinically manifest allergy correlates with LTT responses. In our cohort, the specificity of the LTT for the prediction of chromium allergy was higher than of the patch test [31/41 (76%) *vs*. 25/41 (61%), Fig. 42.11]. Furthermore, the rate of professional exposure to chromium was significantly ($P = 0.01$) higher in sensitized individuals with *versus* without allergy. All other variables included in the questionnaire and examined by the dermatologist did not show any correlation with LTT responses toward chromium.

Interestingly, IFN-γ responses toward chromium chloride were significantly correlated with the strength of patch-test reactivity ($r = 0.49$, $P = 0.002$). But patch-test reactivity showed no correlation with LTT responses. Of note, atopy score, patch-test results, sensitization against other metals, and IFN-γ, IL-2, IL-4, IL-10, and IL-12 production to the ELISpot did not significantly discriminate between sensitization with and without allergy. In addition, the interval from patch test to venipuncture had no impact on the strength of chromium-specific lymphocyte proliferation or cytokine production.

Finally, as depicted in Fig. 42.13, the diagnosis of an allergy against chromium appears as very likely if three criteria are fulfilled: (*i*) professional exposure to chromium, (*ii*) positive patch test (verification of sensitization), and (*iii*) positive reaction to the LTT. In our cohort the combined presence of the three criteria significantly ($P < 0.0001$) predicted allergy with an OR of 54.7 and a specificity of 100% (41/41). Using these close criteria, the sensitivity, however, is low (39%). Thus, the combined evaluation appears as an excellent tool to assure the diagnosis of chromium allergy, for example, in the case of an occupational dermatosis.

DISCUSSION

The core theme of this report is the comparative validation of an LTT and ELISpot assay to analyze nickel- and chromium-specific cellular in vitro responses.

Previously, only few authors have focused on a correlation analysis of nickel-specific LTT and medical history or patch-test results. Either was the group of allergic volunteers studied too small (11) or the proliferative responses were also observed frequently in individuals with negative patch-test results (6,13). Although unusual, we included the medical history into the analysis because a few patients report a typical anamnesis of nickel allergy but do not show positive patch-test reactions and vice versa indicating that also the "gold standard," the patch test, could rarely have problems in detecting nickel allergy. Here, our data indicate that—after optimized assay conditions—the LTT was positive in 95% and 12% of volunteers with or without an anamnesis of nickel allergy, respectively. Similarly, the ELISpot was positive in 87 and 10% of volunteers with and without nickel allergy.

Our data on nickel allergy were in accordance with findings of other groups, also describing by ELISpot technology a specific increase in IFN-γ production in nickel-allergic volunteers (15,36). The results on IL-2 production as determined by ELISpot are furthermore similar to ELISA data on an increased IL-2 secretion in nickel-allergic volunteers (52). In contrast to our own findings, Kapsenberg et al. (53) observed substantial amounts of IL-2 production in nonallergic volunteers as well. Most probably, this difference in cytokine production is attributable to variable assay conditions (e.g., number of responding cells, PBMCs *vs*. T-cell clones, duration of cell culture). Our ELISpot kinetics indicate that the duration of cell culture has great impact on the quantitative pattern of the cytokines produced.

In addition, the frequency of nickel-reactive cells, which we determined by ELISpot (19×10^5 IFN-γ–producing PBMCs) is similar to the results obtained by Cavani et al. (54) who observed by limiting dilution technique a mean precursor frequency of 28×10^5 for $CD4^+$ and 5×10^5 for $CD8^+$ T cells.

Furthermore, the results on nickel toxicity indicate that in patients with high tissue concentrations adjacent to stainless steel implants (43)—for example, observed due to the loosening of an implant—there could be a local, moderate effect on immune function. The reduction of specific lymphocyte proliferation toward tetanus toxoid by 49% indicates that infections in the tissue adjacent to stainless steel implants may be fostered.

Our study on chromium allergy especially focused on the discrimination between volunteers with sensitization with *versus* without allergy. It took into account that the patch test produces a high rate of false positives due to irritant reactions. In our own cohort, 34% of reactions were false positive; other authors even estimate that half of the reactions were false positive (13). Thus, in contrast to previous studies that compared either allergic (30–32) or patch-test–positive volunteers (33) with healthy controls we considered three different groups: with sensitization with allergy, with sensitization without allergy, and nonsensitized healthy controls. We wanted to know if cellular in vitro responses could discriminate between sensitized individuals with and without clinically manifest chromium allergy. We were the first to show that the strength of LTT reactions toward chromium chloride and potassium dichromate was predictive of allergy (17,55). As compared to the patch test, the rate of false positives was lower in the LTT performed in our own cohort. However, in contrast to the LTT, the production of the cytokines IFN-γ, IL-2, IL-4, IL-10, and IL-12 to the ELISpot could hardly predict chromium allergy.

Both trivalent and hexavalent chromium induced proliferation and cytokine production to a similar degree. However, for comparable

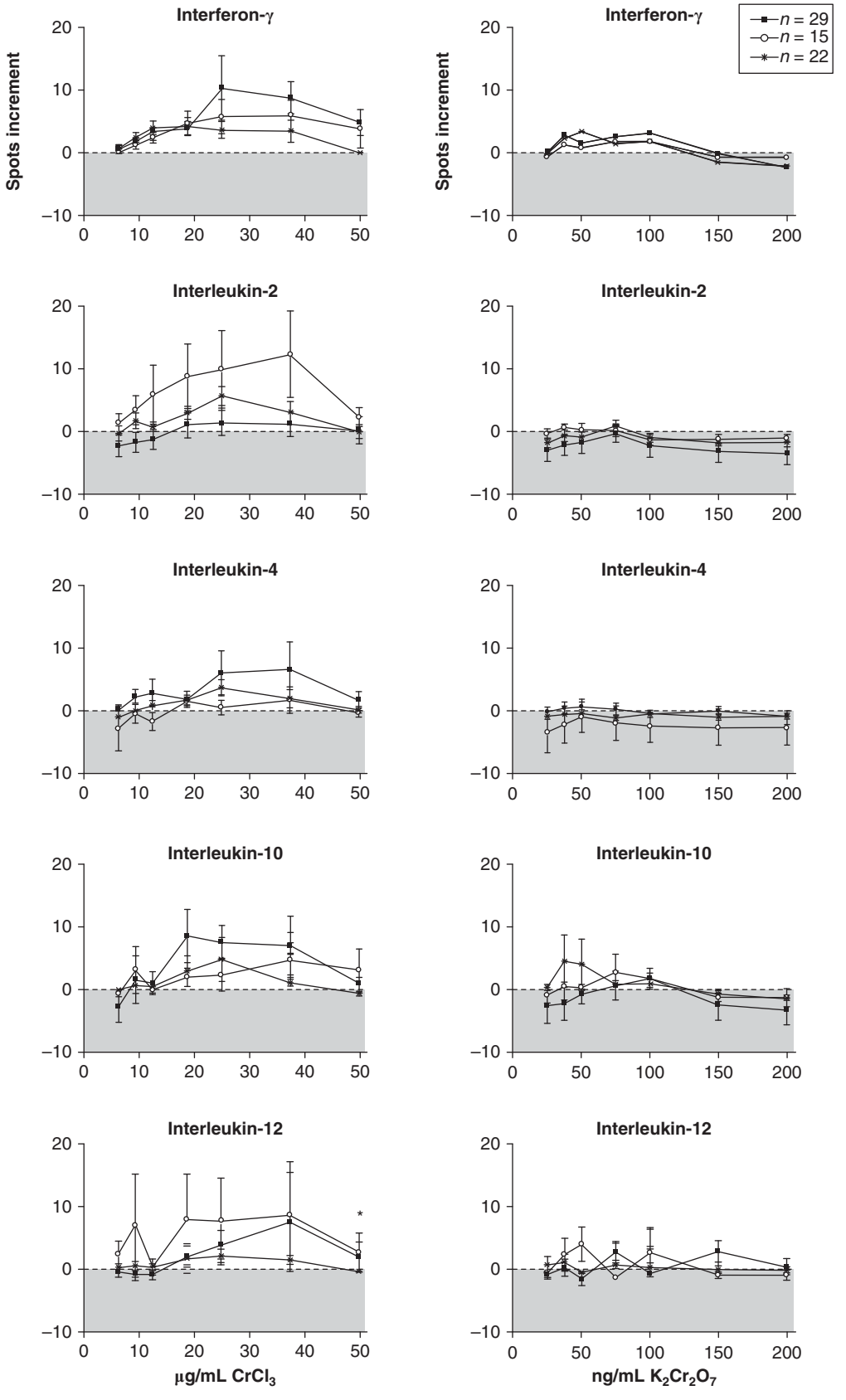

FIGURE 42.12 Dose–response curves of interferon-γ, interleukin (IL)-2, IL-4, IL-10, and IL-12 ELISpot results using seven different concentrations of $CrCl_3$ and $K_2Cr_2O_7$. Data in sensitized volunteers with allergy (filled squares), without allergy (open circles), and nonsensitized healthy controls (asterisk) are given as mean ± standard error of the mean. Toxic reactions toward chromium compounds are shaded in gray. $*P < 0.05$ for sensitized volunteers with allergy *versus* healthy controls (Mann–Whitney *U* test). *Abbreviations*: $CrCl_3$, chromium chloride; $K_2Cr_2O_7$, potassium dichromate.

Criteria for the diagnosis of chromium allergy

	Sensitivity	Specificity
1. Professional exposure 2. Positive patch test 3. Positive LTT	39%	100%

LTT – Confirmation of diagnosis

FIGURE 42.13 Sensitivity and specificity of the combined analysis of professional exposure, positive patch test, and positive lymphocyte transformation test toward chromium ($n = 74$). *Abbreviation*: LTT, lymphocyte transformation test.

results, an approximately 250-fold higher concentration of chromium chloride was needed, compared with potassium dichromate. This weaker T-cell reactivity to chromium chloride can be explained by the earlier findings of Al-Tawil et al. (33) and Räsänen et al. (30), who demonstrated that trivalent chromium binds strongly to proteins, most of which converts to a nonimmunogenic form (56). Apart from the induction of specific lymphocyte proliferation, chromium compounds appear as toxic, a phenomenon, which we observed especially in sensitized volunteers without allergy and healthy controls (Figs. 42.9A and 42.10). Cytotoxicity of tri- and hexavalent chromium on in vitro cell cultures had previously been analyzed (19) and the underlying mechanism reviewed (57). A series of studies indicates that chromium compounds induce oxidative stress through enhanced production of reactive oxygen species leading to genomic DNA damage and oxidative deterioration of lipids and proteins (57).

SUMMARY AND CONCLUSION

Summarizing the data on nickel allergy, patch test, LTT, and ELISpot yielded rather similar results. Thus, in routine testing, cellular in vitro tests are usually not required. However, under certain circumstances it should be performed by specialized laboratories, for example, if patch-test results and anamnesis are inconsistent or if patients are at an increased risk of being sensitized.

On the contrary, the LTT may be superior to the patch test for diagnosing chromium allergy. Here, the ELISpot does not appear as suitable. The chromium LTT is especially valuable to discriminate between chromium-sensitized individuals with and without allergy. It could be defined as an excellent method to confirm the diagnosis of chromium allergy, for example, when an expert opinion is requested due to occupational dermatosis. In combination, an anamnesis of professional exposure, patch test, and LTT could predict chromium allergy with a specificity of 100%.

ACKNOWLEDGMENTS

I thank J. Böhmer, F. Rietschel, and M. Zabel for the recruitment and clinical examination of nickel and chromium allergic volunteers and controls. I am also grateful to H. Haass, M. Huben, and S. Wortmann for their excellent technical assistance, and A. Rüsberg and I. Weigel for their clerical support.

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43 Methods for in vitro skin metabolism studies

Robert L. Bronaugh

INTRODUCTION

It has been known for a number of years that enzymes in skin catalyze a wide variety of metabolic reactions (1–3). All of the major enzymes important for systemic metabolism in the liver and other tissues have been identified in skin (1). Often enzyme activity has been found to be lower in skin (on a per milligram tissue basis) when compared with the liver (4,5). However, given the fact that the skin is the largest organ in the body with a surface area of 2 m^2 and total weight estimated at 4 kg, about three times that of the liver (1), it can play an important role as a portal of entry of chemicals into the body.

Some chemical groups, such as esters, primary amines, alcohols, and acids, are particularly susceptible to metabolism in skin. Many esters are hydrolyzed by esterase to their parent alcohol and acid molecules (6–9). Primary amines are frequently acetylated during absorption through skin (10–12). Oxidation/reduction and conjugation of alcohols and acids are commonly observed in skin (6,10,13).

Chemicals that undergo significant metabolism in skin may exhibit greater or lesser biologic activity than predicted simply from skin penetration studies. A more thorough examination of the safety or efficacy of these compounds can be determined by evaluating the skin absorption and metabolism simultaneously using in vitro techniques.

REASONS FOR DOING IN VITRO STUDIES

Skin metabolism studies are difficult to conduct accurately in vivo because of systemic metabolism that takes place before samples are collected in the blood, urine, or other site. In vitro studies isolate the skin from the metabolic activity in the rest of the body. When studies are conducted using viable skin in diffusion cells, metabolites can be measured in skin homogenates or in the receptor fluid directly beneath the skin. Also, in vitro studies may be the only ethical way to obtain human skin metabolism data for chemicals having safety concerns.

MAINTENANCE OF SKIN VIABILITY IN DIFFUSION CELLS

Human or animal skin should be freshly obtained. Skin previously frozen for shipping or storage is unsuitable for metabolism studies. Enzyme activity with some stable enzymes can sometimes be observed in nonviable skin but the activity may be at a reduced level as noted with esterase activity (6,7).

The viability of rat skin can be maintained for at least 24 hours in flow through diffusion cells using any of the several physiologic buffers as the receptor fluid (14). For 24-hour studies the use of flow-through cells is likely required so that nutrients are continually provided to the skin.

Although a tissue culture media [minimal essential media (MEM)] was satisfactory in maintaining skin viability, it was not required. Simpler balanced salt solutions, such as HEPES-buffered Hanks' balanced salt solution (HHBSS) or Dulbecco modified phosphate-buffered saline worked just as well and are potentially less problematic for analytical reasons. Some of the vitamins, cofactors, and amino acids contained in MEM absorb ultraviolet (UV) light and can interfere with UV detection during high-performance liquid chromatography (HPLC) analysis. Bovine serum albumin (BSA) has been added to the receptor fluid to more closely simulate in vivo conditions, and thereby enhance partitioning of lipophilic test compounds from skin into the receptor fluid.

SKIN VIABILITY ASSAYS

Viability of skin was primarily assessed in our initial studies by measuring aerobic and anaerobic glucose utilization (14). Anaerobic metabolism of glucose to lactic acid predominates in skin, which is why this assay has been commonly used. Glucose is the primary energy source for skin cells and has been monitored by tissue banks to assess skin viability for transplants (15). We also used other techniques to confirm that skin viability was maintained in our diffusion cells. Electron and light microscopy techniques were used to assess viability by demonstrating that the cellular organelles were still intact at the end of 24-hour studies. Skin metabolism of estradiol and testosterone was also maintained for 24 hours.

We have observed that the addition of 4% BSA to HHBSS results in a lowering of lactate levels measured in the skin viability assay (16). Therefore, the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) assay was adapted to assess skin viability when BSA was required in the receptor fluid. The MTT assay of skin viability was not affected by the addition of BSA to the receptor fluid. The viability of human, fuzzy rat, and hairless guinea pig skin was found to be maintained for 24 hours. However, the assay can only be conducted at the end of a study when skin can be removed from the diffusion cell. The lactate measurement of glucose utilization can be conducted during the course of an experiment to provide a more accurate determination of tissue viability throughout the study.

SKIN METABOLISM DURING IN VITRO ABSORPTION STUDIES

Early studies from our laboratory used intact viable dermatomed skin sections from mice, rats, hairless guinea pigs, and humans in flow through diffusion cells to study the penetration and metabolism of estradiol and testosterone (14), Acetyl ethyl tetramethyl tetralin (AETT) and butylated hydroxytoluene (BHT) (4), benzo(a)pyrene and 7-ethoxycoumarin (17), and azo colors (18).

The percutaneous absorption and metabolism of three structurally related compounds, benzoic acid, *p*-aminobenzoic acid (PABA), and ethyl aminobenzoate (benzocaine) were determined in vitro with hairless guinea pig and human skin (10). Approximately 7% of the absorbed benzoic acid was conjugated with glycine to form hippuric acid. Acetylation of primary amines was found to be an important metabolic step in skin. For benzocaine, a molecule susceptible to both *N*-acetylation and ester hydrolysis, 80% of the absorbed material was acetylated, whereas less than 10% of the absorbed ester was hydrolyzed. PABA was much more slowly absorbed than benzocaine and was also less extensively *N*-acetylated. Acetyl-PABA was found primarily in the receptor fluid at the end of the experiments but the receptor fluid contained only 20% of the absorbed dose. Much of the absorbed PABA remained unmetabolized and in the skin as might be expected for an effective sunscreen agent. The compound in skin would probably not have been exposed to *N*-acetylating enzymes if it was localized primarily in the stratum corneum. A similar pattern of benzocaine metabolism was observed in human and hairless guinea pig skin; however, there appeared to be less enzyme activity in human skin.

The effect of benzocaine dose on its absorption and metabolism was determined in the hairless guinea pig (11). It was of interest to determine if metabolism of absorbed benzocaine remained extensive when the radiotracer doses used in our earlier studies were increased to doses simulating human use conditions as a local anesthetic. Percutaneous absorption of benzocaine increased 50-fold when the applied dose increased from 2 to 200 μg/cm^2. Metabolism of benzocaine to acetylbenzocaine was reduced at the higher dose but still approximately one-third of the absorbed dose was metabolized (Table 43.1). The metabolism of benzocaine in skin may not affect the local anesthetic activity of a topical commercial product because benzocaine and acetylbenzocaine were found to have similar potencies in reducing conductance in the isolated squid giant axon (11).

Esterase activity and alcohol dehydrogenase activity were characterized in hairless guinea pig skin with the model compounds methyl salicylate and benzyl alcohol (6). Subsequently, the absorption and metabolism of the cosmetic ingredient retinyl palmitate was determined in human and hairless guinea pig skin.

The metabolism of methyl salicylate was determined in viable and nonviable hairless guinea pig skin. In viable skin over 50% of the absorbed compound was hydrolyzed by esterases in skin to salicylic acid. Twenty-one percent of the absorbed compound was further conjugated with glycine to form salicyluric acid. Greater esterase activity was observed in male skin. Esterase is a stable enzyme and hydrolysis of methyl salicylate also occurred in nonviable skin. However, no conjugation of salicylic acid was observed in nonviable skin.

Oxidation of benzyl alcohol was also observed in hairless guinea pig skin. Approximately 50% of the absorbed benzyl alcohol was oxidized to benzoic acid in viable skin with a small portion of this compound being further metabolized to the glycine conjugate, hippuric acid. As with the ester, a significant activity was also observed in nonviable skin, and greater oxidation of the alcohol was obtained with male skin.

TABLE 43.1
Effect of Benzocaine Dose on Its Metabolism, Percentage Distribution of Benzocaine, and Metabolites in Receptor Fluid and Skin in 24 Hours

	Dose Level		
Location and Compound	**2 μg/cm^2**	**40 μg/cm^2**	**200 μg/cm^2**
Receptor fluid			
Benzocaine	9.6 ± 4.2	50.7 ± 6.6	54.0 ± 5.2
AcBenz	83.8 ± 4.4	43.8 ± 5.7	37.9 ± 3.6
PABA	1.0 ± 0.3	0.1 ± 0.1	0.9 ± 0.5
AcPABA	5.1 ± 1.0	5.8 ± 0.9	7.2 ± 1.7
Skin			
Benzocaine	26.7 ± 14.2	2.4 ± 2.4	62.7 ± 12.2
AcBenz	6.9 ± 6.9	34.4 ± 20.3	20.9 ± 11.7
PABA	4.3 ± 4.3	3.2 ± 3.2	1.5 ± 1.3
AcPABA	24.7 ± 14.9	15.2 ± 16.0	14.9 ± 1.2
Total			
Benzocaine	10.7 ± 3.3	49.9 ± 6.5	57.3 ± 3.7
AcBenz	80.5 ± 3.8	43.6 ± 5.6	34.3 ± 3.4
PABA	1.4 ± 0.2	0.1 ± 0.1	0.9 ± 0.5
AcPABA	6.5 ± 1.0	5.9 ± 1.0	7.6 ± 2.0

Values are the mean ± SE for 1–6 determinations in each of three animals. The 40 μg/cm^2 dose levels values are the mean ± SE of 2–3 determinations in each of four animals. The dosing vehicle was acetone.
Abbreviations: AcBenz, acetylbenzocaine; PABA, *p*-aminobenzoic acid; AcPABA, acetylPABA; SE, standard error.

The absorption and metabolism of retinyl palmitate was measured to see if ester hydrolysis and alcohol oxidation occurred with this cosmetic ingredient. Most of the absorbed radioactivity remained in the skin. A substantial amount of the absorbed compound was hydrolyzed by esterase to retinol but no oxidation of the alcohol to retinoic acid was observed. Any effects of retinyl palmitate on the structure of the skin may be due to the formation of retinol during percutaneous absorption.

Alcohol and aldehyde dehydrogenase conversion of alcohols to acids in rat skin has been reported for a series of alcohols and glycol ethers (13). More than twice the specific activity was obtained in cytosol from dermatomed skin than from full-thickness skin. This suggests that a greater activity for these enzymes resides in the cells of the epidermis. Rates of alcohol dehydrogenase activity in rat skin cytosol decreased in the order 2-butoxyethanol > 2-phenoxyethanol > ethylene glycol > 2-ethoxyethanol > ethanol. Skin penetration studies of 2-ethoxyethanol showed rapid penetration through excised rat skin in vitro but no metabolism of the compound could be detected (19). This suggests that although skin has enzymes capable of metabolizing a chemical, rapid penetration of that chemical through skin may reduce the chance for significant metabolism.

Skin esterase activity has been compared in human, minipig, and rat skin (20). Esterase activity in human and minipig skin microsomes was similar and several orders of magnitude less than that obtained with rat skin microsomes with *p*-nitrophenyl acetate as the substrate. Less esterase activity was observed in plasma and

there was also a smaller difference between human, minipig, and rat skin activity in plasma. Rat skin still had the greatest esterase activity with a 2- to 3-fold increase in activity over human skin.

Absorption values from in vitro studies with viable hairless guinea pig skin have been found to compare closely with in vivo results for phenanthrene (21) and for pyrene, benzo[a]pyrene, and di(2-ethylhexyl) phthalate (22). Also, significant metabolism was observed in vitro during the absorption of all four compounds.

Phenanthrene was metabolized in vitro to 9,10-dihydrodiol, 3,3-dihydrodiol, 1,2-dihydrodiol, and traces of hydroxyl phenanthrenes (21). Following topical administration of phenanthrene, approximately 7% of the percutaneously absorbed material was converted to the dihydrodiol metabolites.

Numerous metabolites of benzo[a]pyrene were formed during percutaneous absorption through hairless guinea pig skin (22) Of particular interest was the identification of benzo[a]pyrene 7–10 tetrahydrotetrol in the diffusion cell receptor fluid. This metabolite is the hydrolysis product of the ultimate carcinogen, 7,8-dihydroxy, 9,10-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene. This study demonstrates that skin metabolism is likely responsible for skin tumors formed following topical benzo[a]pyrene administration. In the earlier phenanthrene study (21), no known carcinogenic metabolites were formed during skin penetration. This finding is consistent with the lack of tumorigenicity of phenanthrene in rodents.

Since the systemic toxicity of topically applied compounds is sometimes evaluated by the oral route of administration, the effect of route of administration on metabolism of (^{14}C) 2-nitro-*p*-phenylenediamine (2NPPD) was examined in vitro in the fuzzy rat (12). Rat skin dermatomed to approximately 250 μm and full-thickness rat intestinal tissue (from the jejunum) were assembled into flow-through diffusion cells perfused with HHBSS to maintain viability. 2NPPD was applied for 30 minutes to the skin in a semi-permanent hair dye formulation and to intestine in HHBSS (pH 6.5). A similar amount of radioactivity was absorbed into the receptor fluid from each tissue during the 24-hour studies.

The metabolism of 2NPPD was determined in receptor fluid fractions using a HPLC method. More than 50% of the 2NPPD applied to skin remained unmetabolized, whereas only 40% of 2NPPD was unmetabolized by intestine (Fig. 43.1). Subsequently, more acetylation of 2NPPD to N4-acetyl-2NPPD occurred during absorption through skin. However, triaminobenzene was formed to a greater extent in intestine. The amount of sulfated 2NPPD and/or metabolites (actual compound or compounds not determined) was also greater in effluent from intestinal tissue. The extent of metabolism of 2NPPD in human skin (semipermanent hair dye vehicle) was also determined. Approximately 60% of the absorbed radioactivity was metabolized to equal amounts of triaminobenzene and N4-acetyl-2NPPD. No sulfated compounds were found in effluents from human skin. These studies showed significant differences in metabolism during absorption through human and rat skin as well as differences in metabolism through rat skin and intestinal tissue.

The skin absorption and metabolism of arachidonic acid (AA) and glyceryl arachidonate (GA) were assessed in diffusion cells following application in an emulsion vehicle (8). The skin absorption of AA was 19.5% and 52.3% of the applied dose in human and rat skin, respectively, with most of the absorbed material remaining in the skin at the end of the studies. Similar rapid absorption was obtained for GA through human skin. It was also

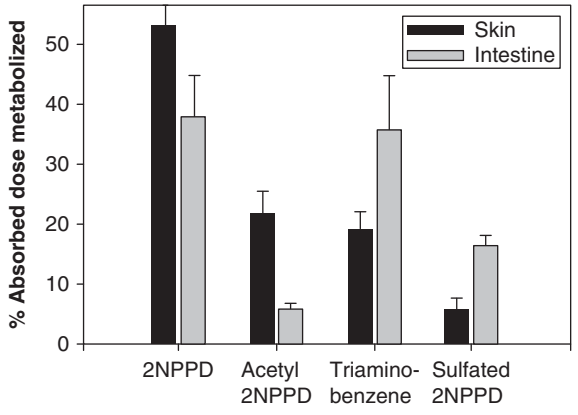

FIGURE 43.1 Percentage of the absorbed dose of 2NPPD metabolized in rat skin and intestinal tissue. Values are the mean ± SE from three studies. Solid lines, skin tissue; pale lines, intestinal tissue. *Abbreviation*: 2NPPD, 2-nitro-*p*-phenylenediamine.

of interest to determine whether the glyceryl arachidonate ester could be hydrolyzed to AA. Only small amounts of unmetabolized GA could be identified by HPLC in the human skin homogenates prepared at the end of the 24-hour studies. AA formation was 27.8% of the absorbed dose and 42% of the radiolabeled material had been converted to likely metabolites of AA during skin penetration. It therefore appears that substantial amounts of AA would be formed following topical application of GA in a lotion to human skin.

Absorption and skin metabolism of catechin compounds from green tea extract alone or administered in chitosan microparticles has recently been examined (23). Skin absorption was assessed using neonatal human foreskin in diffusion cells. Skin metabolism of catechins was examined separately following a 24-hour incubation with chopped foreskin in phosphate-buffered saline. The chitosan microparticle carrier was found to significantly improve the skin absorption of epicatechin, epigallocatechin, and epigallocatechin gallate while also preventing metabolic degradation of these compounds.

Rapid skin absorption of *N*-methyl-2 pyrrolidone (NMP) was found through rat skin by both in vivo and in vitro techniques. However, no metabolism of NMP was observed during a 2-hour in vitro study in either the receptor fluid (physiologic buffer) or skin homogenates (24).

The toxicity of topically applied D&C red no. 17 (PAN) is of concern because of potential metabolism of the color to the carcinogenic compound 4-aminoazobenzene. Radiolabeled PAN was applied to excised human and pig skin assembled in diffusion cells. Less than 1% of the applied dose was found in the receptor fluid indicating a low potential for systemic absorption. No metabolism of PAN was observed in viable pig skin using HPLC techniques (25).

CONCLUSIONS

Metabolism of chemicals in skin can be determined by in vitro diffusion cell techniques using viable skin. This method can provide important information about the activity or toxicity of a chemical that penetrates into or through the skin. Data with human skin is more relevant for safety investigations. Skin metabolism can also be measured in skin homogenate preparations (cytosol,

microsomes) made from human or animal skin. These studies are useful in determining the types of enzyme activity present in skin and for comparing the rank order of metabolism of a series of chemicals. But these types of studies cannot replace diffusion cell studies in determining the extent of metabolism of a chemical during percutaneous absorption.

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44 In vitro model for decontamination of human skin: Formaldehyde*

Hongbo Zhai, Xiaoying Hui, and Howard I. Maibach

Chemical injuries are commonly encountered after exposure to acids and alkali, including hydrofluoric acid, formic acid, anhydrous ammonia, cement, and phenol. The concentrations of corrosive agents, potency, and duration of their contact primarily determine the degree of skin damage. Decontamination of a chemical from skin is the reduction or removal of chemical agent. Decontamination may be accomplished by removal of these agents by physical means or by chemical neutralization or detoxification. Tregear (1) initiated this field in the 1940s, but practical interventions remain limited.

Immediately after exposure to such chemicals, washing off with water alone or together with soap, is a traditional measure to reduce damage and minimize percutaneous penetration. Wester et al. (2) reported that removing alachlor with water was less effective than with soap and water. A traditional soap-and-water wash and the emergency water shower are relatively ineffective at removing methylene bisphenyl isocyanate, a potent contact sensitizer, from skin (3). Thus, water, or soap and water, may not be the most effective means of skin decontamination, particularly for lipophilic materials. In some cases, chemical left on the skin after traditional washing procedures can have toxic consequences (4). Further development of robust decontamination agents is indicated.

Formaldehyde, used widely by industry, is a common allergen and irritant (5). This was chosen as a model low-molecular weight compound—and is considered only as such. Thus, the interpretation is for this aldehyde—and not for all chemicals. This study compared the capacity of decontamination solutions using in vitro model system on human skin.

MATERIALS AND METHODS

Contaminant

Aqueous solution of radiolabeled [14C]-formaldehyde (0.1 mCi/mL; specific activity: 51.9 mCi/mmol) was purchased from Sigma–Aldrich Company (St. Louis, MO, USA).

Model Decontamination Solutions

Isotonic saline (0.9%; pH 5.94) and hypertonic saline (1.8%; pH 5.71) were obtained from VWR International (West Chester, PA, USA). Tap water (pH 8.09) was taken from faucet. The source is the Hetch Hetchy reservoir (Sierra Nevada Mountains, CA, USA).

Human Skin

Human cadaver skin was obtained from the Northern California Transplant Bank and dermatomed to 500 μm thickness. Skin samples were stored in Eagles Minimum Essential Media with Earle's balanced salt solution (BSS) (In Vitro Scientific Products Corp., St. Louis, MO, USA) and refrigerated at 4°C prior to use within five days after death to maintain viability (6–8). Five skin samples were used.

Procedure

Skin was placed onto glass diffusion cells with rubber bands. The cells had been prefilled with a maximum amount of the receptor fluid (0.9% sodium chloride), approximately 6 mL in each. Then, aliquots 10 μL (approximately 0.25 μg) of [14C]-formaldehyde solution was dosed by a high-performance liquid chromatography syringe onto each skin surface. After a defined exposure time (1, 3, and 30 minutes postdosing, respectively), the surface skin (3 cm^2) was washed with 4 mL of each solution per time; a total of 12 mL of each solution was used to wash off one skin. All washing liquids were collected individually into a scintillation glass vial for radioactive measurement. The skin was then stripped with tape disks (D-Squame®, Cuderm Corporation, Dallas, TX, USA) twice (removing residua of chemical on the skin superficial). Lastly, the wash solutions, strippings, receptor fluid, and rest of the skin were counted to determine the amounts of [14C]-formaldehyde.

Evaporation Test

This test monitored the [14C]-formaldehyde percentage evaporation at exposure times of 1, 3, 15, 30, and 60 minutes, respectively. Plastic disks (1.75 cm in diameter and 0.178 mm thick) were each applied with 1 μCi/10 μL/cm^2 of [14C]-formaldehyde and timed for the appropriate duration of exposure. Triplicates for each time point were taken giving a total of 15 disk samples. Then each disk sample was immediately placed into its designated scintillation vial and filled with scintillation cocktail.

Scintillation Counting

The scintillation cocktail was UniverSol™ (MP Biomedicals, Costa Mesa, CA, USA). Background control samples and the test samples were counted in a computer-controlled liquid scintillation

*Adapted and updated from Zhai H, Barbadillo S, Hui X, Maibach HI. In vitro model for decontamination of human skin: formaldehyde. Food Chem Toxicol 2007; 45: 618–621, with permission from Elsevier.

analyzer (Tri-Carb® 2900TR, Perkin–Elmer, Inc., Wellesley, MA, USA). Control and test sample counts transferred to a computer program that subtracted background control samples. The inhouse counting process and the computer program have been verified to be accurate by a quality assurance officer.

Statistical Analysis

Statistical analysis was performed using a computer program SigmaStat® (SPSS Science, Chicago, IL, USA). Differences were analyzed using the one-way repeated measures ANOVA. Statistical significance was accepted at $P < 0.05$.

RESULTS

The mean values, standard deviations (SDs), and percent dose (%) of [14C]-formaldehyde obtained from each group are summarized in Table 44.1. There were no statistical decontaminating differences among those groups except that isotonic saline, at 3 minutes postexposure (in wash solutions), showed a significant ($P < 0.05$) difference (87%) when compared with tap water (62.6%) (Table 44.1 and Fig. 44.1).

Formaldehyde percentage evaporation increased linearly ($R^2 = 0.94$) with extended application times (Fig. 44.2). Evaporation = 10.8 + 0.433 time; the percentage evaporation of formaldehyde are 7.7%, 13.6%, 19.7%, 24.4%, and 35.9% (1, 3, 15, 30, and 60 minutes, respectively).

TABLE 44.1
Decontamination Capacity to [14]C-Formaldehyde Post-Topical Administration of (a) 1 min (b) 3 min, and (c) 30 min on Human Skin In Vitro

	Tap Water	Isotonic Saline	Hypertonic Saline
(a) 1 min			
In wash solutions	0.207 ± 0.049 (82.6%)	0.220 ± 0.033 (88.2%)	0.245 ± 0.031 (97.8%)
In tape strips	0.001 ± 0.001 (0.5%)	0.001 ± 0.000 (0.5%)	0.002 ± 0.001 (0.6%)
In receptor fluid	0.003 ± 0.001 (1.4%)	0.000 ± 0.000 (0.1%)	0.001 ± 0.001 (0.3%)
In skin	0.003 ± 0.010 (1.2%)	0.001 ± 0.004 (0.5%)	0.002 ± 0.012 (0.8%)
Total recovery	85.7%	89.2%	99.6%
(b) 3 min			
In wash solutions	0.156 ± 0.030 (62.6%)	0.217 ± 0.033* (87.0%)	0.185 ± 0.018 (73.8%)
In tape strips	0.006 ± 0.002 (2.2%)	0.005 ± 0.002 (1.8%)	0.005 ± 0.002 (2.0%)
In receptor fluid	0.001 ± 0.001 (0.2%)	0.004 ± 0.006 (1.5%)	0.001 ± 0.001 (0.2%)
In skin	0.008 ± 0.005 (3.2%)	0.007 ± 0.003 (2.9%)	0.005 ± 0.001 (2.1%)
Total recovery	68.2%	93.2%	78.2%
(c) 30 min			
In wash solutions	0.104 ± 0.043 (41.7%)	0.072 ± 0.020 (28.6%)	0.101 ± 0.045 (40.3%)
In tape strips	0.016 ± 0.005 (6.6%)	0.020 ± 0.009 (8.1%)	0.016 ± 0.010 (6.3%)
In receptor fluid	0.002 ± 0.002 (1.0%)	0.001 ± 0.001 (0.4%)	0.002 ± 0.001 (1.0%)
In skin	0.036 ± 0.012 (14.4%)	0.027 ± 0.020 (10.9%)	0.031 ± 0.012 (12.5%)
Total recovery	63.6%	48.0%	60.1%

Data were shown as mean ± SD of mass (μg) and percent dose (%).

DISCUSSION

Previous study (2) indicated that skin decontamination of alachlor at 0 hours with soap and water removed 73% ± 15.8% ($n = 4$) of the applied dose with the first wash; this increased to a total of 82.3% ± 14.8% with two additional washes. Decontamination after 1 hour removed 87.5% ± 12.4% with three successive washes. After 3 hours the decontamination ability decreased, and after 24 hours only 51.9% ± 12.2% could be recovered with three successive washes. Using water only, at 0 hours 36.6% ± 12.3% alachlor was removed with the first wash and the total increased to 56.0% ± 14.0% with two additional washes. At 24 hours the total amount decreased to 28.7% ± 12.2% for three successive washes. Continual successive washes (6–8 in sequence) recovered 80–90% of the skin-applied alachlor.

In the current study, at all time points, most formaldehyde was recovered in the wash solutions. By 3 minutes, only isotonic saline provides a statistically significant enhanced decontamination compared with tap water. By 30 minutes, significance was lost. The flux (receptor fluid and some of the skin sample) must be interpreted differently than usual, as the diffusion cells were taken apart at 1, 3, and 30 minutes, and not followed for the typical 8–24 hours. Experiments should be done in which the cells are followed for 24 hours. Results showed that three model decontamination solutions were almost equally effective in removal of the applied dose of formaldehyde. However, isotonic saline provided a slight enhancement.

The volatility of the applied dose from the skin was determined and showed a linear trend with extending application time. Even with the rapid formaldehyde evaporation, penetration into skin occurred within 30 minutes post application. As noted, obtaining total mass balance proved difficult presumably because of volatility. Our mass balance accountability was 48–99.6% compared with a previous study in vivo with a less volatile chemical where the dose accountability was 80.6–95.2% (2). Thus, we conducted a study utilizing a less volatile contaminant (glyphosate) to improve this decontamination model and the results indicate that mass balance accountability is 94.8–102.4% (9).

Hui et al. (10) and Wester et al. (11) developed a high-throughput model using ground callus and delipidized callus, noting a relationship of binding to callus and penetration. Relating the callus assay to the current model may be of value as both in vitro methods provide facile system that might aid prediction of decontamination assessment. Based on solubility of contaminate and decontamination solution, pH, volume of decontamination, time of removal ratios, physical enhancements (such as stripping, rubbing, and adhesives defining binding properties), robust decontamination agents/systems may be developable. For instance, a recent controlled experiment shows that water rinsing followed by topical calcium provided favorable results for hydrofluoric acid skin decontamination (12). Nielsen (13) used four model compounds (benzoic acid, glyphosat, caffeine, malathion) with varying size

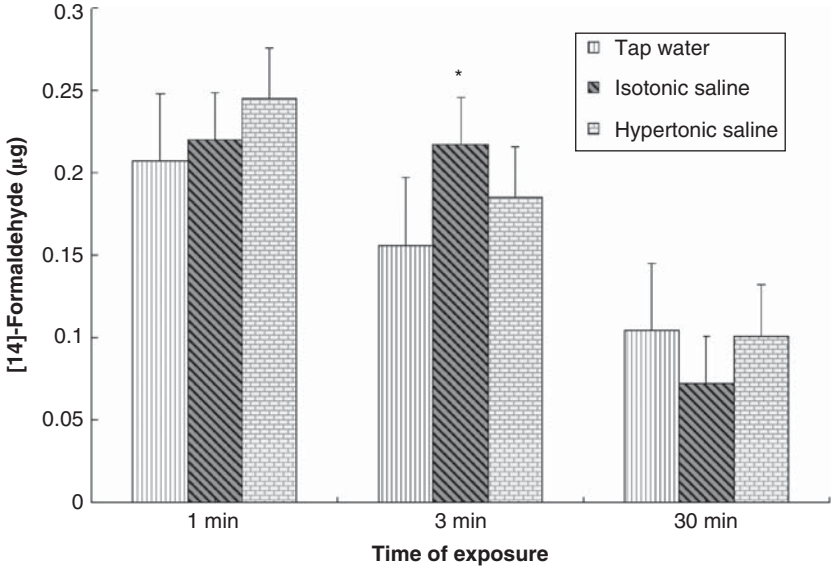

FIGURE 44.1 Formaldehyde in wash solution. Data were shown as mean ± SD of mass (µg).

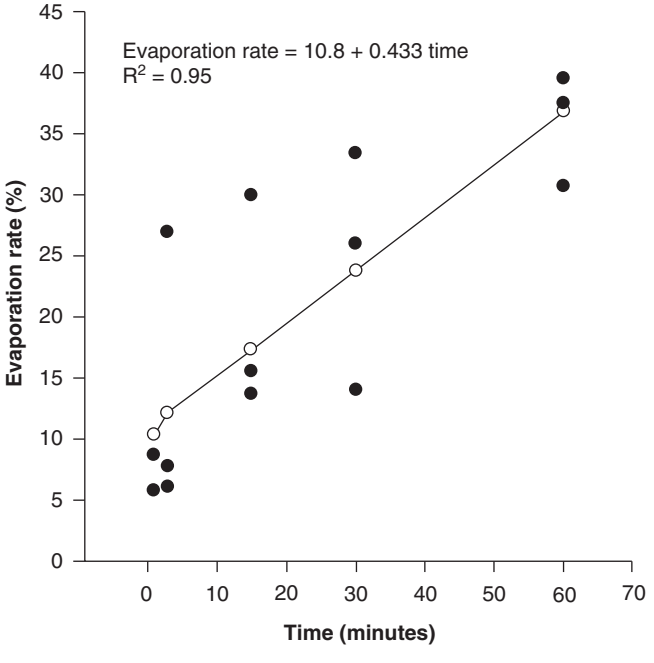

FIGURE 44.2 Formaldehyde evaporation test. • (closed circle) observed individual evaporation; o (open circle) and – (line) predicted evaporation.

and solubility to quantify the effect of skin wash after 6 hours of dermal exposure in an in vitro model with static diffusion cells. The study demonstrated that percutaneous penetration continues after end of exposure due to the reservoir present in the skin. However, penetration rate will decrease significantly, and it is evident that simple hand-wash after end of exposure not only reduces the amount of residue present in the upper skin compartment but also significantly reduces the total absorption of test substance, most so for the hydrophilic compounds. Note, however, the important details provided by Hall et al. (14), as these may have significantly influenced these results. They emphasized the immediate use of skin decontamination agent as a critical factor of efficacy after hydrofluoric acid exposure. The view was confirmed with experimental observations (15,16). Recently, an active decontamination solution for chemical splash injuries has shown promising results in an in vitro study (17). In addition, adoption of splashing or acute burning models may also dramatically result in diametrically opposite outcomes with decontamination agents' potency. Chan et al. (18) reviewed pesticide percutaneous absorption and decontamination. It is important to understand percutaneous absorption of a tested chemical as an integral part of the risk assessment process. Additionally, the "wash-in" effect should pay particular attention because it may act as an enhancement of percutaneous absorption by action of skin decontamination that might be brought into skin of the washing chemicals to become bioavailable via the systemic cutaneous blood supply (19).

To-date, practical interventions still remain limited. New decontamination modalities, such as containing neutralizer or against a broad range of chemical agents, require refinement and validation. This model might be used as a rapid screening procedure in the development of effective decontamination agents. Yet, the in vitro human skin model may—when validated with other chemicals and subsequent in vivo verification (2,3)—provide more rapid enhancement of our practical knowledge in this complex field.

Taken together, this is one small step—to eventually developing practical and effective evidence-based decontamination systems. Hui and Maibach (20) provide extensive documentation of mechanistic insights that might aid these developments.

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45 Percutaneous absorption of hazardous substances from soil and water

Josephine Gerby, Ronald C. Wester, and Howard I. Maibach

INTRODUCTION

Contamination and transfer of hazardous chemicals in soil and water (ground and surface water) is a major concern. When the large surface area of the skin is exposed to contaminated soil and water (during work, play, swim, daily bath, etc.), skin absorption may be quite significant. Brown et al. (1) suggested that skin absorption of contaminants in water has been underestimated and that ingestion may not constitute the sole, or even the primary exposure route. Soil has become an environmental depository for potentially hazardous chemicals, which is why the important question about how to extrapolate experimental measurement to different soil contaminated at different concentration and with different skin contact remains relevant for risk assessors. Déglin et al., (2), provided a model to predict the soil saturation limit on chemical (Ssoil) assuming the contribution on both the surface absorption and the organic carbon absorption. Exposure through work in pesticide-sprayed areas on chemical dump sites seems obvious. However, there may be hidden dangers in weekend gardening or in children's play area. This chapter demonstrates the potential risk from contaminated soil and water, and discusses the potential error in dependence on model systems without validation.

PERCUTANEOUS ABSORPTION

Solvents

Numerous sites have significant levels of organic contaminants in soil, which are either slowly released or degraded, providing a potential long-term source for chemical exposures. Remediation clean-up cost varies dramatically with the level to which soil must be decontaminated. However, a difficulty in establishing soil clean-up level stems, in part, from our lack of knowledge of the dermal bioavailability of chemicals following exposure to environmental media. Compared with dermal exposures with neat or aqueous compound, little is understood about the dermal bioavailability of solvents in soil, dust, sludge, or sediment matrices. A method has been developed to determine dermal uptake of solvents under non-steady state conditions using real-time breath analysis in rats, monkeys, and human volunteers. The exhaled breath was analyzed using an ion-trap mass spectrometer, which can continually quantitate chemicals in the exhaled breath stream in the 1–5 ppb range. The resulting exhaled breath data were evaluated using physiologically based pharmacokinetic (PBPK) models to estimate dermal permeability constants (Kp), under various exposure conditions.

Exposures have been conducted comparing the impact of exposure matrix (soil vs. water), occlusion versus nonocclusion, and species-differences on the percutaneous absorption of methyl chloroform, trichloroethylene (TCE), and pentachloroethylene. Studies have demonstrated that rat skin may be 40 times more permeable than human skin (20,21), that bioavailability is decreased when exposures are in a soil versus aqueous matrix, and that under nonoccluded exposure conditions the majority of the compound is lost to volatilization and is unavailable for absorption. These results have clearly illustrated that the methodology was sufficiently sensitive to enable the conduct of animal and human dermal studies at low exposure concentrations over small body-surface areas, for short periods of time.

Table 45.1 summarizes PBPK estimates for solvent human in vivo dermal absorption. Hand immersion treatment is a volunteer sitting comfortably with his/her hand immersed in a bucket of water or soil containing one of the solvents. The volunteer wears a face-mask. The volunteer inhales fresh air from an air tank. The mask has a special device that switches between inhalation and exhalation.

Thus, the volunteer exhales through a different pathway such that the exhaled breath goes to a tandem ion-trap mass spectrometer (MS/MS) coupled to a computer that records and can display real-time (every few seconds if wanted) the solvent concentration in the exhaled breath (3–5).

Table 45.2 gives PBPK model estimates for the dermal absorption of TCE in rats. Estimated permeability constants are listed. Generally, solvent dermal absorption is less for humans than for rats. In both species, solvent absorption is less from soil than from water. This may be due to water's ability to retain solvent within a matrix on the skin better than with soil. The combination of real-time breath analysis and PBPK modeling provides an opportunity to effectively follow the changing kinetics of uptake, distribution, and elimination phases of a compound throughout a dermal exposure. The sensitivity of the ASGDI-MS/MS system for exhaled-breath analysis is pivotal in enabling studies wherein human volunteers are exposed to low levels of compounds for short periods of time. This real-time, in vivo method is suitable for studying the percutaneous absorption of volatile chemicals, and allows exposures to be conducted under a variety of exposure conditions, including occluded versus nonoccluded, rat versus monkey versus human, and soil versus water matrices (6).

Organic Chemicals

DDT, Benzo[A]Pyrene, Chlordane, Pentachlorophenol, and 2,4-D

Table 45.3 gives the in vitro (human skin) and in vivo (Rhesus monkey) percutaneous absorption of organic chemicals from soil and a

TABLE 45.1
PBPK Model Estimates for Human in Vivo Dermal Absorption

Solvent	Treatment	K_p (cm/h)
Methylchloroform (TCA)	Water hand immersion	0.0063 ± 0.0006
Perchlorolthylene (PCE)	Soil hand immersion	0.019 ± 0.001
	Water patch	0.0074 ± 0.000
	Soil patch	0.0043 ± 0.002
Trichloroethylene (TCE)	Soil hand immersion	0.0009 ± 0.0003

TABLE 45.2
PBPK Model Estimates for the Dermal Absorption of TCE in Rats

Exposure concentration	K^a_p (cm/h)	Amount absorbed (mg)	Total TCE recovered[b]
Occluded water[c] (mg/L)			
1600	0.31 ± 0.018	7.5 ± 1.4	100 ± 5.2
600	0.30 ± 0.006	2.7 ± 0.4	103 ± 5.1
Average	0.31 ± 0.014	102 ± 5.6	
Nonoccluded soil[c] (mg/kg)			
40,600	0.087 ± 0.002	1.5 ± 1.4	98 ± 8.8
20,300	0.085 ± 0.003	7.3 ± 2.7	97 ± 5.7
5000	0.085 ± 0.003	1.7 ± 0.8	101 ± 1.4
Average	0.086 ± 0.003	99 ± 6.0	
Occluded soil[c] (mg/kg)			
15,600	0.090 ± 0.003	40 ± 15	99 ± 2.2
5300	0.089 ± 0.002	14 ± 3.7	99 ± 2.4
Average	0.090 ± 0.002	99 ± 1.0	

[a]Water K_p values are significant from soil ($P < 0.01$) for bolh occluded and nonoccluded studies. "There is no significant difference in K_p between occluded and nonoccluded soil exposures.

[b]The total TCE recovered was calculated from percent absorbed (estimated from PBPK. model), percent remaining in media (soil or water), and percent in charcoal path, where appropriate (as measured using GC head-space analysis), ± SD.

[c]The amount absorbed by the body for nonoccluded soil exposure is for a three hours exposure ($n = 3$), ± SD.

TABLE 45.3
In Vitro and In Vivo Percutaneous Absorption of Organic Chemicals

Compound	Vehicle	Skin	Percent dose in vitro receptor Fluid	In vivo
DDT	Acetone	18.1 ± 13.4	0.08 ± 0.02	18.9 ± 9.4
	Soil	1.0 ± 0.7	0.04 ± 0.01	3.3 ± 0.5
Ben[a]pyrene	Acetone	23.7 ± 9.7	0.09 ± 0.06	51.0 ± 22.0
	Soil	1.4 ± 0.9	0.01± 0.06	13.2 ± 3.4
Chlordane	Acetone	10.8 ± 8.2	0.07 ± 0.06	6.0 ± 2.8
	Soil	0.3 ± 0.3	0.04 ± 0.05	4.2 ± 1.8
Pentachlorophenol	Acetone	3.7 ± 1.7	0.6 ± 0.09	29.2 ± 5.8
	Soil	0.11 ± 0.04	0.01 ± 0.00	24.4 ± 6.4

TABLE 45.4
Octanol/Water Partition Coefficients of Compounds

Compounds	LogP
DDT	6.91
Benzo[a]pyrene	5.97
Chlordane	5.58
Pentachlorophenol	5.12
2,4-D	2.81
PCBs	Mixture
Aroclor l242	(High logP)
Aroclor 1254	(High logP)

comparative vehicle (water or solvent, depending on vehicle). The soil is from the same source (Yolo County) for all chemicals. For each chemical the concentration of mass (μg) per unit skin area (cm^2) is the same for each vehicle. Note that chemical selection was done according to chemical interest, as expressed by Cal EPA and US EPA. Thus, the chemicals exhibit high logP octanol/water partition coefficients, rather than a range of logPs (Table 45.4).

In vivo human skin percutaneous absorption is expressed as chemical percent dose in receptor fluid accumulation and skin content. Chemicals with higher logPs are lipophilic and, therefore, are not soluble in biological fluid or receptor fluid (plasma, buffered saline) (7–9). Receptor fluid (human plasma) accumulation of DDT was negligible in the in vitro study due to solubility restriction. Human skin content was 18.1% dose from acetone vehicle. In vivo absorption in the rhesus monkey was 18.9% dose from acetone vehicle. These values are comparable with the published 10% dose absorbed in vivo in human from acetone vehicle. Percutaneous absorption from soil was predicted to be 1.0% dose in human skin in vitro and a comparative 3.3% dose in vivo in rhesus monkey. In vivo percutaneous absorption of benzo[a]pyrene is high—51.0% reported here for rhesus monkey (19) and 48.3% (10) and 35.3% (11) for rat. Benzo[a]pyrene absorption from soil was approximately one-fourth that of solvent vehicle (7). For chlordane, pentachlorophenol, and 2,4-D, the in vivo percutaneous absorption in rhesus monkey from soil was equal to or slightly less than that obtained from the solvent vehicle (Table 45.3). Validation to human in vivo is available for 2,4-D where the percutaneous absorption is the same for rhesus monkey and human. In vitro percutaneous absorption is variable, probably due to solubility problems relative to high lipophilicity.

PCBS

Table 45.5 gives the in vitro and in vivo percutaneous absorption of PCBs (12). As with the other organic chemicals with high logP, receptor fluid accumulation in vitro was essentially nil. Skin accumulation in vitro did exhibit some PCB accumulation. In vivo, PCB percutaneous absorption for both Aroclor 1242 and 1254 was (1) high, ranging from 14% to 21%, and (2) generally independent of formulation vehicle. Thus, PCBs have a strong affinity for skin and are relatively easily absorbed through skin. Figure 45.1 summarizes absorption from solvent and soil.

Metals

Arsenic, Cadmium, and Mercury

Selected salts of arsenic, cadmium, and mercury are soluble in water, and thus are amenable to in vitro percutaneous absorption

TABLE 45.5
In Vitro and In Vivo Percutaneous Absorption of PCBs

Compound	Vehicle	Skin	Percent dose in vitro receptor fluid	In vitro
PCBs (1242)	Acetone	–	–	21.4 ± 8.5
	TCB	–	–	18.0 ± 8.3
	Mineral oil	6.4 ± 0.3	0.3 ± 0.6	20.8 ± 8.3
	Soil	1.6 ± 0.1	0.04 ± .05	14.1 ± 1.0
PCBs (1254)	Acetone	–	–	14.6 ± 3.6
	TCB	–	–	20.8 ± 8.3
	Mineral oil	10.0 ± 16.5	0.1 ± 0.07	20.4 ± 8.5
	Soil	2.8 ± 2.8	0.04 ± 0.05	13.8 ± 2.7

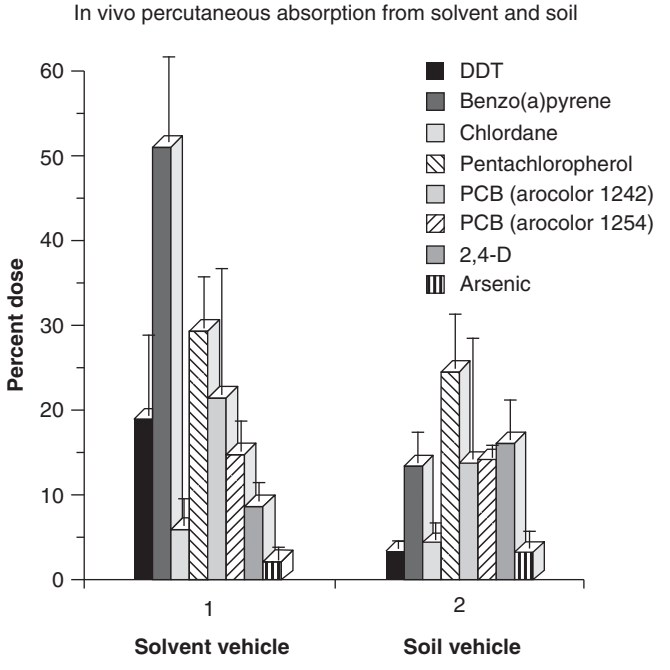

FIGURE 45.1 In percutaneous absorption of several hazardous substances from soil and solvent (either acetone or water), overall, soil reduced absorption to about 60% compared with solvent. However, the absorption of some compounds is the same for soil and solvent.

TABLE 45.6
In Vitro and in Vivo Percutaneous Absorption of Metals

Compound	Vehicle	Skin	Percent dose in vitro receptor fluid	In vivo
Arsenic	Water	1.0 ± 1.0	0.9 ± l.l	2.0 ± 1.2
	Soil	0.3 ± 0.2	0.4 ± 0.5	3.2 ± 1.9
Cadmium	Water	6.7 ± 4.8	0.4 ± 0.2	–
	Soil	0.09 ± 0.03	0.03 ± 0.02	–
Mercury	Water	28.5 ± 6.3	0.07 ± 0.01	–
	Soil	7.9 ± 2.2	0.06 ± 0.01	–

TABLE 45.7
In Vitro Receptor Fluid Versus In Vivo Percutaneous Absorption

Compound	Vehicle	Percent dose in vitro receptor fluid	In vivo
DDT	Acetone	0.08 ± 0.02	18.9 ± 9.4
	Soil	0.04 ± 0.01	3.3 ± 0.5
Benzo[a]pyrene	Acetone	0.09 ± 0.06	51.0 ± 22.0
	Soil	0.01 ± 0.06	13.2 ± 3.4
Chlordane	Acetone	0.07 ± 0.06	6.0 ± 2.8
	Soil	0.04 ± 0.05	4.2 ± 1.8
Pentachlorophenol	Acetone	0.6 ± 0.09	29.2 ± 5.8
	Soil	0.01 ± 0.00	24.4 ± 6.4
PCBs(1242)	Acetone	–	21.4 ± 8.5
	TCB	–	18.0 ± 8.3
	Mineral oil	0.3 ± 0.6	20.8 ± 8.3
	Soil	0.04 ± 0.05	14.1 ± 1.0
PCBs(1254)	Acetone	–	14.6 ± 3.6
	TCB	–	20.8 ± 8.3
	Mineral oil	0.1 ± 0.07	20.4 ± 8.5
	Soil	0.04 ± 0.05	13.8 ± 2.7
2,4-D	Acetone	–	2.6 ± 2.1
	Soil	0.02 ± 0.01	15.9 ± 4.7
Arsenic	Water	0.9 ± 1.1	2.0 ± 1.2
	Soil	0.03 ± 0.5	3.2 ± 1.9
Cadmium	Water	0.4 ± 0.2	–
	Soil	0.03 ± 0.02	–
Mercury	Water	0.07 ± 0.01	–
	Soil	0.06 ± 0.01	–

with human skin. Arsenic absorption in vitro was 2.0% (1.0% skin plus 0.9% receptor fluid), and the same in vivo in rhesus monkey. Absorption from soil was equal to (in vivo) or approximately one-third (in vitro). Cadmium and mercury both accumulate in human skin, and are slowly absorbed into the body. (Note that in vivo studies with cadmium and mercury are difficult to perform; cadmium accumulates in the body and mercury is not excreted via urine.) Note the high skin content with cadmium and mercury (Table 45.6) (8,13).

IN VITRO DIFFUSION VERSUS IN VIVO

Regulatory agencies have developed an affinity for a calculated permeability coefficient (Kp) for risk assessment. Permeability coefficients are easily determined from the time course of chemical diffusion from a vehicle (water, soil) across the skin barrier into a receptor fluid. Table 45.7 compares in vitro diffusion receptor fluid absorption with in vivo percutaneous absorption. Receptor fluid accumulation for the higher logP chemicals (Table 45.4) is negligible. This is due to basic chemistry—the compounds are not soluble in the water-based receptor fluid. Based on these receptor-fluid accumulations these chemicals are not absorbed by the skin. Risk assessment would contain an extreme false-negative component. That point where the diffusion system and receptor fluid accumulation gives a true Kp or manufactures a false Kp has not been determined. Regulatory agents should have some in vivo validation before blindly accepting an in vitro Kp.

SOIL LOAD

A popular assumption is that only the fine particles of soil, which stick to the skin transfer contaminants from the soil to the skin.

TABLE 45.8
Effect of Soil Load on 2,4-D Percutaneous Absorption

System	Soil load[a] (mg/cm)^2	Percent dose absorbed[b]
In vivo, rhesus monkey	1	9.8 ± 4.0[c]
	40	15.9 ± 4.7
In vitro, human skin	5	1.8 ± 1.7
	10	1.7 ± 1.3
	40	1.4 ± 1.2

[a]Concentration of 2,4-D chemical per cm^2, skin area was kept constant, while soil load per cm^2 skin area was varied.
[b]In vivo percutaneous absorbtion measured by urinary ^{14}C accumulation; in vitro absorption measured by urinary ^{14}C skin content.
[c]Mean ± SD (n = 4).

This is the monolayer theory. If it was only the fine soil particles, then all of the data shown in this chapter could not exist, because the fine particles were not used (sieved out for laboratory personnel safety reasons). Besides, contaminants will transfer between large surfaces (table, couch, etc.) and skin, even between people. And, certainly, the first soil monolayer to contact the skin during planting of the first rose bush will not be the same monolayer after planting the twentieth bush. However, the computer model needs the monolayer, therefore, it has to exist.

Approaches by various research groups have varied. Spalt et al. (14) used chemicals added to the soil in a volatile solvent that was removed by evaporation. But the investigated contaminant concentration and soil loads substantially exceeded those that would occur in an environmental exposure. However, it is established that in aqueous suspensions, the equilibrium concentrations of organic compounds in soil are proportional to their aqueous concentration. Lyman et al. (15) based that organic matter content of the soil reported as the mass fraction of organic carbon per mass of soil (foc). After this consideration, it has been proposed that dermal absorption should be inversely proportional to foc (14,16,17). Based on this and Goss et al's. (18) equation ($S_{soil} = f_{oc} S_{oc} + \sigma_{soil} M_{\sigma}$) we could expect that for a given soil, the amount of chemical that will absorb into the skin increases proportionally with the concentration of chemical in the soil, as long as the soil concentration is less than the soil saturation concentration.

Déglin et al. (2) studied different soils and chemicals, as a result, saturation concentration varied with soil surface area in addition to the amount of soil organic matter. Spalt also provides a summary and commentary on the experimental literature as of 2009.

Table 45.8 shows the effect of soil load. Note the chemical concentration was kept constant while soil load varied.

DISCUSSION

The evolution of skin resulted in a tissue that protects precious body fluids and constituents from excessive uptake of water and contaminants from the external environment. The outermost surface of the skin for humans is the stratum corneum, which restricts but does not prevent penetration of water and other molecules. This is a complex lipid–protein structure that is exposed to contaminants during bathing, swimming, and exposure to the environment. Industrial growth has resulted in the production of organic chemical and toxic metals whose disposal resulted in contamination. When an adult settles into a tub or a when child plays in dirt, the skin (the largest organ of the body), acts as a lipid sink (stratum corneum) for the lipid-soluble contaminants.The skin also serves as transfer membrane for water and whatever contaminants that are dissolved in it. It is most important to note that (1) water transfers through skin and can carry chemicals, and (2) the outer layer of skin is lipid in nature. Thus, highly lipophilic chemicals such as DDT, PCBs, and chlordane residing in soil will quickly transfer to skin. Percutaneous absorption can be linear, orderly, and predictive (a measured flux from water). However, evidence exists that chemicals may transfer to skin with short-term exposure. Regulators should be cautious as in vitro and computer models are developed for risk assessment. Validation is needed to avoid false-negative assessment.

At the present, when a soil is contaminated the characteristics that are affecting soil saturation are confusing. Only when this is clarified, will protocols be created on contaminated soil that will be closest to the actual dermal exposures.

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46 Stratum corneum tape-stripping method: An update

Yue Zheng, Myeong Jun Choi, Hongbo Zhai, and Howard I. Maibach

INTRODUCTION

Tape stripping is useful for removing stratum corneum (SC) and in obtaining information about the function of this thin layer as a main barrier for skin penetration. Typically, an adhesive tape is pressed onto the test site and is subsequently abruptly detached. The number of tape strips need to remove the SC varies with age, sex, and possibly ethnicity.

Tape stripping has been used in dermatological and pharmaceutical fields to measure the SC mass and thickness (1–3), to investigate percutaneous penetration and disposition of topically applied drug in vivo (4–7), and to disrupt skin barrier function (6–8). Also, this technique has been used to collect SC lipid samples (9), to detect proteolytic activity associated with the SC (10), and to quantitatively estimate esterase activities in the SC (11). Tape stripping is a quantitative and minimally invasive assay for the detection of metal on and in the skin (12). Tape stripping has been used to disrupt the skin before percutaneous peptide (protein) and DNA immunization (13,14).

Tape stripping is of sufficient utility to have been proposed by the FDA as part of a standard method to evaluate bioequivalence of topical dermatological dosage forms (15). Ikeda et al. (15) reported the cutaneous bioavailability of topically applied maxacalcitol ointment in vivo by tape stripping. Tape stripping is simple and inexpensive; it has been most frequently used for investigation of skin penetration, barrier function, and defining factors in skin pathology. In addition, tape stripping is fast and easy to use in human studies.

This chapter reviews the method, considering factors, analytic method of drug in the SC after stripping, and its application on the penetration enhancement into SC and topical vaccination and summarizes recent data.

STRATUM CORNEUM AND ITS FUNCTIONS

SC is a stratified squamous epithelium lining the body surface that plays an important antidesiccating role as a barrier function and a reservoir for topically applied substances (16). SC consists of nonviable cornified cells (corneocytes) embedded in lipid-rich intercellular domains (intercorneocyte spaces). Intercellular domains comprise free fatty acids (FFAs), cholesterol (CHOL), and ceramides (CERs), together with smaller amounts of cholesteryl sulfate, sterol, triglycerides, squalene, n-alkanes, and phospholipids. Nine different extractable CERs have been detected in human SC, which are classified as CER1 to CER9. The CER can be subdivided into three main groups, based on the nature of their head group architecture (sphingosine, phytosphingosine, or 6-hydroxysphingosine).

SC lipids localize mainly in the intercellular space with little in the corneocytes (17). These lipids exist as a continuous lipid phase; occupying about 20% of the SC volume, arranged in multiple lamellar structures. All CERs and fatty acids found in SC are rod and cylindrical in shape; this physical attribute makes them suitable for the formation of highly ordered gel phase membrane domains. CHOL is capable of either fluidizing membrane domains or of enhancing rigidity, depending on the physical properties of the other lipids and the proportion of CHOL relative to the other component. Intracellular lipids that form the only continuous domain in the SC are required for a major competent barrier.

Efforts have been undertaken to characterize the lipid lamellar regions. X-ray diffraction studies on native SC demonstrate that the SC lipids are organized in two coexisting crystalline lamellar phases: the short periodicity phase with a periodicity of approximately 6 nm and the long periodicity phase (LPP) with a periodicity of approximately 13 nm (18). The LPP and its predominantly orthorhombic lipid packing are considered crucial for the skin barrier function. SC lipids, CER, CHOL, and FFA form the orthorhombic lateral packing, a densely packed structure. However, in equimolar mixtures prepared for CHOL and CER, the major lipid fraction forms a lamellar phase (hexagonal lateral packing) with periodicity of 12.8 nm. Addition of FFA to CER/CHOL mixtures induced a transition from a hexagonal to orthorhombic lateral packing (19). Therefore, the formation of the characteristic LPP depends on the presence of CHOL and specific CER, in particular CER1, whereas FFAs are required for the crystalline (orthorhombic) character of the lateral lipid packing (18,20).

Diseases such as atopic dermatitis, psoriasis, and contact dermatitis are associated with barrier dysfunction. Most skin disorders that have a diminished barrier function present a decrease in total CER content with some differences in their pattern (21). Pilgram et al. (22) reported that in the case of diseased skin, an impaired barrier function is related to an altered lipid composition and organization. In atopic dermatitis SC, they found that, in comparison with healthy SC, the presence of the hexagonal lattice (gel phase) is increased with respect to the orthorhombic packing (crystalline phase). From lipid composition studies of atopic skin, it has been found that intercellular lipids, especially CERs, play an important role in the barrier function and lipid organization.

Man-Qiang et al. (23) suggested that for the formation of a component SC barrier, the CER, CHOL, and FFA should be present in an equimolar ratio. It has also been suggested (24) that three major SC lipids are required for permeability barrier homeostasis and the equimolar composition of major lipids is increased up to threefold for acceleration of barrier repair. Barrier repair creams including natural components of SC lipids have been used to treat skin disease (25,26). Chamlin et al. (25) reported a phase I trial of a repair cream in childhood atopic dermatitis.

The physiology of SC may differ between genders. Only a few studies on gender-related differences in skin physiology have been performed and they provided conflicting results (27,28). Jacobi et al. (29) investigated the effect of gender on SC. The skin of women was characterized by a significantly higher pH value (5.6 ± 0.4) than that of men (4.3 ± 0.4, $p < 0.05$). There were no significant differences between women and men volunteers in transepidermal water loss (TEWL), SC hydration, and sebum content. Protein absorption was the only other parameter significantly dependent on gender. The difference of skin pH and protein absorption might be caused by differences in human biology, such as hormonal status.

SC REMOVAL METHODS AND EFFECT OF STRIPPING

To remove SC, tape stripping for mechanical removal of corneocytes and solvent-extraction method to remove both polar and nonpolar SC lipids are used. Tape stripping is a useful technique for selectively removing the skin's outermost layer, while solvent extraction is a delipidization process in SC.

In general, a clinical description of the barrier disruption differs depending on the disruption methods. For tape-stripped skin, the typical description was moderate erythema and a glistening surface due to total removal of the SC; for acetone-treated skin, the description was minimal or no erythema and slight superficial dryness; and for chloroform–methanol mixture, the description was deep erythema and edema (7). Thus, an organic solvent method using chloroform–methanol mixing may be more aggressive than the standard tape.

The change of skin condition after stripping differs depending on stripping (Table 46.1). Fluhr et al. (8) investigated the barrier recovery pattern after tape stripping or acetone delipidization at five body sites in healthy volunteers. The fastest barrier recovery after tape stripping and acetone delipidization was observed on the forehead, followed by the back. However, there are differences in SC capacitance values following acetone and tape stripping. In the case of acetone delipidization, there were no statistically significant differences in SC capacitance between body sites. In contrast, tape stripping produces significant differences in capacitance values between body sites. The capacitance increases are related to strong barrier damage by tape stripping. However, the decrease of capacitance appears related to lipid extraction. Benfeldt and Serup (6) reported that salicylic acid penetration was greatly increased with the tape stripping, but not with acetone in the skin of hairless rats.

After barrier disruption, there are typically no adverse effects, such as infection or scarring. However, disruption of permeability barrier by tape stripping induces activation and maturation of epidermal Langerhans' cells (30). This process is important in inducing immune response in vivo and in immunizing with peptide and protein by a percutaneous method.

TABLE 46.1
Physiological Changes of the Human and Rat Skin after Stripping

		Barrier	Tape	
Type of Skin	**Perturbation**	**None**	**Stripping[a]**	**Acetone[b]**
Human	TEWL (g /cm^2/h)[c]	4.3 ± 2.2	30.6 ± 22.2	9.1 ± 7.5
	Erythema (arbitrary unit)[d]	8.7 ± 2.8	11.6 ± 2.8	9.2 ± 1.5
Rat	Δ TEWL[e]	0	69 ± 14	6 ± 3
	Δ Erythema[e]	0	2.41 ± 0.87	0.95 ± 1.66

[a]Tape stripping was achieved by applying 2.5 × 5 cm (human) and 5 × 5 cm (rat) piece of Transpore tape with firm pressure and repeating the procedure 20 (human) and 10 (rat) times, respectively.

[b]Acetone was treated by gentle wiping with large cotton buds soaked in 100% acetone for 3 min.

[c]Fifteen minutes after barrier perturbation procedures, TEWL was measured using an evaporimeter and recorded in triplicate.

[d]Colorimetry measures skin color by analyzing the light reflected from the skin surface according to the standardization protocol for the content of green-red (a^*) and yellow-blue (b^*) color and skin brightness (L^*). The a^* redness parameter is a measure of erythema.

[e]TEWL and erythema from the barrier perturbed skin area minus the value from the untreated side.

Data from Ref. 6.

STRIPPING FACTORS

When tape stripping is employed, the following factors are important: (*i*) number of strips, (*ii*) types and size of tapes, (*iii*) the pressure applied to the strip prior to stripping and the peeling force applied for removal, and (*iv*) the anatomic site. Some parameters are summarized in Table 46.2. We summarize the effect of the type of tape and the number of strips.

Dreher et al. (2) improved the method by quantifying the amount of human SC removed by each strip utilizing a colorimetric protein assay. With this method, Bashir et al. (1) determined the physical and physiological effect of SC tape stripping, utilizing tapes with different physicochemical properties. Three commercial adhesive tapes utilized were D-Squame® (CuDerm, Dallas, Texas, USA), Transpore® (3M, St Paul, Minnesota, USA; batch no. 2002–12 AP), and Micropore® (3M, batch no. 2001–08 AN). D-Squame is precut into disc shape. Transpore and Micropore are provided as a standard roll. Table 46.3 shows the components of three commercial adhesive tapes and the effect of tapes on the TEWL) depending on the number of strips. Bashir et al. (1) demonstrated that no significant difference was found in the kinetic parameters (mean water diffusion coefficient, SC thickness, and permeability) between the tapes. However, there are differences in the mean TEWL values. Mean TEWL increased

TABLE 46.2
Comparison to Tape-Stripping Methods

Type of Tape	Stripping Number	Size	Applied Pressure	Time	Study
D-Squame	40	14 mm	10 Kpa	2 s	(1)
Transpore	40		10 Kpa	2 s	(1)
Micropore	40		10 Kpa	2 s	(1)
D-Squame	16	14 mm	80 g/cm^3	5 s	(5)
Leukoflex	18–20	7.5 cm^2	Soft pressure		(9)
3M invisible	7		Controlled condition	10 s	(31)
Adhesif 3M 6204	10	20 cm^2		2 s	(11)
Scotch Book tape 845	20		By rubbing six times		(32)
Scotch	7		1-kg-pressure		(33)
Scotch 600	2–5	4 cm	By rubbing		(34)
Blenderm 3M	6	4 cm^2			(35)
Transpore	20	12.5 cm^2	Firm pressure		(7)
Transpore	10	5 × 5 cm			(6)
Teasfilm	20	4 cm^2			(8)
D-Squame	20	3.8 cm^2	Uniform pressure	5 s	(2)
D-Squame	16	25 mm	0.365 N/cm^2		(36)
D-Squame	25	25 mm	Uniform pressure	5 s	(37)
D-Squame	20	2.2 cm	10 Kpa pressure	2 s	(38)
Tesa	20	3.0 cm^2	2-kg-pressure	10 s	(39)
Tesa	26–28	1.9 cm	15 g–25 g/cm^2	15 s	(40)
Cover-Roll tape	5	10 cm^2	Uniform pressure	2 min	(41)
Scotch Book tape 845	20	2.0 cm^2	Uniform pressure		(42)
					(43)

Note: Tape stripping is employed with different adhesive tapes, sizes, number of strips, and pressures applied to the strip prior to stripping and the peeling force applied for removal.

significantly in the deeper layers of the SC reached by tape stripping for the D-Squame and Transpore, but not for the Micropore. Therefore, D-Squame and Transpore tapes induce a significant increase in the TEWL, while Micropore tape did not (Table 46.3). The value of TEWL differed depending on the type of tapes and the number of tape strips. Löffler et al. (44) investigated the influences of stripping procedures (anatomical site, pressure, pressure duration, and tape removal rate) inherent in each stripping protocol on changes in skin physiology. They reported that stripping results were influenced dramatically by all investigated parameters.

The number of tape strips to remove SC differs by investigators and experimental methods such as in vivo and in vitro assay (Table 46.2). The FDA guideline recommends 10 tape strips after topical application of a substance. Weerheim and Ponec (9) reported that the average number of tapes in vivo could be 18–20 strips. For some individuals, 40 adhesive tape strips, regardless of the type of tapes, do not disrupt the SC barrier to water (1). Thus, we consider the factors such as the types of tape and number of strips when applying this method.

TAPE STRIPPING VS. PERCUTANEOUS ABSORPTION AND PENETRATION

Percutaneous absorption and penetration is a complex physical and physiological process. This process initiates a series of absorption and excretion that are influenced by numerous factors. Percutaneous absorption of a drug depends mainly on the permeability coefficient of the drug, which is affected by drug polarity, molecular size, the vehicle in which the drug is applied, and the skin barrier. Other important factors are application conditions (non-occlusion or occlusion) and skin integrity, which is affected by disease and trauma, body site, and age (4,45–47).

The intracellular lipid domain is a major pathway for permeation of most drugs through the SC and also acts as a major barrier for penetration. As a consequence of its hydrophobic nature, the SC barrier allows the penetration of lipid-soluble molecules more readily than water-soluble drugs. Generally, small, nonpolar, lipophilic molecules are the most readily absorbed, while high water solubility confers less percutaneous absorptive capacity through normal skin (47). The way to overcome the properties of the corneal layer is by disrupting it with physical methods (ultrasound, low- and high-voltage electrical pulsing, and stripping) and chemical enhancers.

The tape-stripping method is widely used to measure drug concentration and its concentration profile across the SC. The SC is progressively removed by serial adhesive tape stripping and consequently, percutaneous absorption and penetration was significantly increased in stripped skin (Table 46.4). Benfeldt and colleagues (6,7) reported that in microdialysis experiment salicylic acid was highly increased in tape-stripped skin in human and hairless rats at 157- and 170-fold, respectively. Morgan et al. (47) reported that in microdialysis experiment tape stripping increased penciclovir absorption by 1300-fold and acyclovir absorption by

TABLE 46.3
Components of Three Commercial Tape and Precise TEWL (g /m2/h) Data per Number of Strips for Three Common Tapes at Dorsal Forearm Site

Number of Strips	Type of Tape		
	D-Squame	Transpore	Micropore
Components	Polyacrylate ester, super clear polymer	Iso-octyl acrylate, methyl acrylic acid copolymer	Iso-octyl acrylate, acrylic acid copolymer
Baseline	10.3	8.78	9.37
10	11.23	10.77	8.88
20	14.15	14.12	10.1
30	21.05	21.12	10.4
40	30.33	31.98	13.4

Note: The tape was applied to the test site with forceps and pressed onto the skin with a standardized 10 Kpa pressure for 2 s. The pressure was then removed and the tape was peeled from the skin unidirectionally.
Abbreviations: TEWL, transepidermal water loss.
Data from Ref. 1.

440-fold. Although tape stripping increased the penetration of drugs into the skin, this is not universal (48–50). Physiological and pathological factors affect drug transport across the living human skin. Bos and Meinardi (51) suggested the 500 Da rule for the skin penetration of chemical compounds and drugs. This size limit may be changed by skin abnormalities such as atopic dermatitis and disrupted skin.

Abla et al. (57) reported the effect of iontophoretic current on the acetaminophen and kyotorphin (peptide) delivery across intact and tape-stripped porcine ear skin. Passive permeation of acetaminophen and kyotorphin across intact porcine ear skin was negligible. After removal of the SC, there was a significant increase in passive permeation of acetaminophen (294 ± nmol/cm^2/h) and kyotorphin peptide (98 ± 31 nmol/cm^2/h). However, the application of an iontophoretic current across tape-stripped skin did not result in a further increase in acetaminophen (266 ± 71 nmol/cm^2/h) and kyotorphin (100 ± 30 nmol/cm^2/h). Iontophoretic studies into the transdermal delivery of lidocaine by Sekkar et al. (58) and tacrine by Hirsch et al. (59) across intact and tape-stripped skins have also observed the similar result as Abla et al. (57). From these results, application of iontophoretic current in the impaired skin did not increase transdermal delivery of applied drugs. In addition to organic drugs, tape striping increased the penetration of biological macromolecules such as peptide and DNA into viable skin (13,14). Topically applied oligonucleotides (ONs) and DNA do not penetrate normal human SC. However, removal of SC by tape stripping led to extensive penetration of ONs and DNA throughout the epidermis. Regnier et al. (55) compared ONs' penetration through intact and stripped hairless rat skin. Stripping increased ONs' concentration by one or two orders of magnitude (24- to 166-fold increase) (Table 46.4). In case of plasmid DNA, Yu et al. (60) reported that transfer gene activity depends on the number of stripping. They applied a cytomegalovirus chloramphenicol acetyltransferase expression plasmid to stripped area and found that the transfer gene expression was higher in the murine skin samples stripped five times prior to DNA application compared with those stripped three times prior to DNA application. This result indicated that abrasion of the skin prior to DNA application could improve cutaneous gene transfer and expression. Taken together, tape stripping is a commonly used method to enhance the delivery of chemical drugs and biological macromolecules.

The determination of penetration pathways of topically applied drugs into the skin has been evaluated. However, a direct and noninvasive quantification of the amount of topically applied drugs penetrated into the hair follicular was not available. Teichmann et al. (61) reported differential stripping method to determine the amount of topically applied drug penetrated into the hair follicles. They used differential stripping techniques with a tape stripping and a cyanoacrylate skin surface biopsy. Tape stripping was used to remove the part of the SC that contained the topically applied dye. Subsequently, the follicular contents were ripped off by cyanoacrylate skin surface biopsy. Differential stripping technique is a new method that can be used to study the penetration of topically applied substances into the hair follicular infundibula noninvasively and selectively.

TAPE STRIPPING VS. ANALYTIC METHODS

To determine the drug concentration and profile into SC, analytical techniques are important. These techniques include skin extraction measurement, horizontal stripping and sectioning, quantitative autoradiography, mass spectrometry, optical microscopy, and spectroscopic methods. Penetration into SC is determined by tape stripping followed by skin extraction and spectroscopic methods. These methods are widely used in the determination of drug concentration within skin. Skin extraction is necessary to extract the drug with a suitable solvent and then an appropriate, sensitive analytical method, such as HPLC, spectroscopy, and scintillation counting, is used to quantify the extracted drug. The improving sensitivity of optical instrument has permitted the quantification of drugs in skin by spectroscopic methods. These methods are noninvasive and offer real-time data on penetrated drug localization. These techniques include ATR-FTIR spectroscopy, fluorescence spectroscopy, remittance spectroscopy, confocal microscopy (laser-scanning microscopy), mass spectrometry, and photothermal spectroscopy (16,62,63). Table 46.5 shows the characterization of the analysis method of drugs in the skin.

Tape stripping and optical spectroscopy are used as a suitable combined method to determine the honey layer profile (15,16,40, 62,122,123). The application of tape stripping in combination with analytical instruments (mass spectrometry, UV/VIS spectroscopy, and microscopy) is checked to determine the local position of topically applied substances inside the SC and the penetration profile (15,16). The combined use of these analytical methods can test the validity of the dermatopharmacokinetic method to assess bioequivalence and bioavailability of topical dermatological drugs.

In addition to drug detection methods, many methods detect metal passing into and on the skin: inductively coupled plasma-atomic emission spectroscopy (ICP-AES), inductively coupled plasma-mass spectrometry (ICP-MS), atomic absorption spectrometry (AAS), and particle-induced x-ray emission (PIXE) are widely used. ICP-AES permits detection of metals at the trace amount level, obviating the use of radioisotopes. ICP-MS is a technique applicable to microgram per liter (ppb) concentration of several elements in aqueous medium upon appropriate sample preparation of biological materials. AAS is the reference method

Table 46.4
In Vivo Drug Penetration Studies in Barrier-Perturbed Skin

Barrier Perturbation	Species	Drug	Penetration Ratio[a]	Study
None			1	
Tape stripping	Human	Hydrocortisone	2	(46)
	Human (occulsion)	Hydrocortisone	32.7	(46)
	Human	Low molecular weight heparin	1	(50)
	Human	Methylprednisolone aceponate	91.5	(52)
	Human	Salicylic acid	157	(7)
	Human	Penciclovir	1300	(47)
	Human	Aciclovir	440	(47)
	Hairless guinea pig	Benzoic acid	2.1	(49)
	Hairless guinea pig	Hydrocortisone	3	(49)
	Rat	Nicotinic acid	10.8	(53)
	Rat	Cortisone	2.5	(53)
	Rat	Salicylic acid	0.8–46	(54)
	Hairless rat	Oligonucleotide	24–166	(55)
	Hairless rat	Salicylic acid	170	(6)
	Hairless mouse	Nitroglycerin	9.0	(56)
	Hairless mouse	Enoxacin	7.5	(45)
	Hairless mouse	Biphenylacetic acid	1	(48)
	Porcine ear	Acetaminophen	290	(57)
	Porcine ear	Kyotorphin	100	(57)

[a]Penetration ratio varies among drugs and species investigated. Most of the studies used traditional radiolabeling and HPLC techniques. In case of salicylic acid, the study defined the cutaneous penetration and systemic absorption during 20 min intervals over a period of 4 h after drug administration.

accepted by the International Union of Pure and Applied Chemistry for trace element analysis. PIXE analysis with a proton microprobe allows the determination of trace elements in epidermal strata prepared by cryosection.

TAPE STRIPPING VS. TOPICAL VACCINATION

Why is the skin a major target for topical vaccination? The skin, an active immune surveillance site, is rich in potent antigen-presenting dendritic cells (DCs) such as Langerhans cells (LCs) in the epidermis. LC plays a key role in the immune response to antigenic materials. Skin accessibility makes it an easy target for vaccination. Thus, skin is an attractive target site for topical vaccination and has become the focus of intense study for the induction of antigen-specific immune responses (65–67). Wang et al. (68) observed that protein penetrates SC barrier following occlusion by patch application, but immune responses generated in this way are Th2-predominant. Th2 immune response (humoral immune response) mainly induces antibody production to neutralize soluble antigen and this immune response does not elicit cytotoxic T lymphocytes (CTL) response induced by Th1-dominant immune response that is important in preventing and therapy against viral infections and tumors.

In addition to disruption of the epidermal barrier, stripping enhances in vitro the T-cell-mediated immune response (30). Tape stripping is immunostimulatory and results in the production and release of IL-1α, IL-1β, TNF-α, IL-8, IL-10, and INF-γ (30). Skin barrier disruption by tape stripping also increases co-stimulatory molecule expression (CD86, CD54, CD40, and MHC class II) and the antigen-presenting capacity of epidermal DCs (69). In addition, tape stripping facilitates the generation of Th1 immune responses and stimulates LCs migration to cutaneous lymph nodes (69).

Seo et al. (13) reported that topical application of tumor-associated peptide onto the SC barrier disrupted by tape stripping in mice induces a protective antitumor response in vivo and in vitro. They investigated induction of CTL response on tape-stripped earlobes of C57/BL6 mice by application of CTL epitope peptide onto the SC. The optimal condition for a CTL response was observed 12 and 24 hours after tape stripping at peptide doses of 48 and 96 μg per mouse. However, CTL induction was virtually absent when peptide was applied to intact skin (Table 46.6). Kahlon et al. (69) reported optimization of topical vaccination for the induction of CTL with peptide and protein antigens. They found that tape stripping significantly enhanced antigen-specific antibody (protein) and CTL responses (peptide and protein) measured at three and two weeks following immunization, respectively (Table 46.6). Stripping resulted in prolonged CTL responses at least two months after single immunization. Godefroy et al. (67) reported the systemic and mucosal antibody responses to protein after its application onto the intact or tape-stripped skin. Application of protein antigen alone onto the intact or tape-stripped skin did not elicit any detectable antibody response. These results are inconsistent with Kahlon et al. (69). However, when cholera toxin was used as an adjuvant, good antigen-specific responses (systemic and mucosal) were measured in mice immunized with protein applied onto the tape-striped skin but not onto the intact skin. These results suggest that stripping can be widely used in inducing immune responses with topical vaccination in vivo.

In addition to peptide and protein antigen, tape stripping increased the humoral and cellular immune responses of topical DNA antigens (14). Comparing the immune response with and without stripping, topical application without stripping induced

TABLE 46.5
Description of the Techniques Available for Quantifying Drugs in the Skin

Technique	Penetrants Detected	Measuring Depth	Cost	Speed	Complementary Strategies
Skin extraction	Any	All strata	Inexpensive	Rapid	Separation of skin tissue Qualitative autoradiography
Horizontal sectioning	Any	All strata	Inexpensive	Rapid	Separation of follicles Use of follicle-free skin
Quantitative autoradiography	Radiolabelled only	All strata	Expensive	Slow	None
ATR-FTIR spectroscopy	IR-absorbing only	SC	Expensive	Very rapid	Tape stripping Separation of follicles
Direct fluorescence spectroscopy	Self-fluorescent only	All strata	Medium	Rapid	Quantitative fluorescent microscopy
Indirect fluorescence spectroscopy	UV-absorbing only	SC	Medium	Rapid	None
Remittance spectroscopy	UV-absorbing only	SC	Medium	Rapid	None
Photothermal spectroscopy	Strong UV-absorbing	SC	Medium	Rapid	None
Spectroscopy	UV/Visible	SC	Inexpensive	Rapid	Tape stripping
Microscopy	Common or laser	SC	Medium	Rapid	Tape stripping
Mass spectrometry	Metal	SC	Expensive	Rapid	Tape stripping

Data from Refs. 62–64.

Table 46.6
Comparison to CTL Activity of Peptide, Protein, and DNA Immunization with and without Stripping

Antigen	Immunization	Specific Lysis (%)
Peptide	Intact skin	11.0
	Stripped skin[a]	80.0
	Stripped skin + cholera toxin[b]	70.0
Protein[c]	Intact skin	8.0
	Stripped skin	46.0
DNA[d]	Intact Skin	12.7
	Stripped skin	37.0

[a]Cervical lymph node cells (effectors) obtained from mice immunized 10 days earlier with tyrosinase-related protein 2 peptide (VYDFFVWL, 96 μg per mouse) either through intact earlobes or earlobes tape stripped 12 hr earlier were subjected to CTL assay using Lkb target cells pulsed with tyrosinase peptide. CTL assays were performed at effector-to-target ratio of 10.

[b]C57BL/6 mice were immunized on the ear with 25 μg ovalbumin peptide (SIYRYYGL) and 25 μg cholera toxin following tape stripping. Mice were boosted in similar fashion at 1 week and sacrificed at 2 weeks. Ovalbumin expressing EG7 cells were used as target and CTL assays were performed at effector-to-target ratio of 50. The ear skin on the dorsal and ventral side was tape-stripped 10 times (using Scotch Brand 3710 adhesive tape).

[c]C57BL/6 mice were immunized on the ear with 250 μg ovalbumin protein and 25 μg cholera toxin following tape stripping. Mice were boosted in similar fashion at 1 week and sacrificed at 2 weeks. Ovalbumin expressing EG7 cells were used as target and CTL assays were performed at effector-to-target ratio of 50. The ear skin on the dorsal and ventral side was tapestripped 10 times (using Scotch Brand 3710 adhesive tape).

[d]BALB/c mice were immunized with plasmid DNA coded influenza M protein. Lymphoid cells from each immunized group were restimulated for 5 days using influenza M peptide-pulsed syngenic spleen cells. The peptide pulsed p815 cells were used as targets. CTL assays were performed at effector-to-target ratio of 80. Fast-acting adhesive glue (Alon Alfa®) was smeared on a glass slide to cover the mouse. After an interval of 20–30 s, the slide was ripped off.

weak antibody response and did not elicit a sufficient CTL response. In contrast, topical application of this vaccine with stripping induced strong antibody responses and elicited substantial CTL responses. There was a significant difference between the results of topical application with and without stripping (65,66).

To confirm the protective effect of topical vaccination, teams of researchers (13,14) used an influenza and a melanoma mouse model. Watabe et al. (14) investigated the efficacy of a topical DNA vaccine that expressed the matrix gene of the influenza virus using a mouse model. They topically applied plasmid DNA onto the stripped skin on days 0, 7, and 14. After the third immunization, mice were challenged with 5LD50 of influenza virus; 13 of 20 mice (65%) survived when they were topically immunized with plasmid DNA that expressed the matrix gene. When the mice were immunized with inactivated virus topically, only 18% of mice were protected and all mice were dead seven days after virus inoculation in case of unimmunized control group. These results suggest that the topical administration of DNA vaccine induce a protective immunity against influenza challenge. Seo et al. (13) investigated the efficacy of topical peptide vaccination for tumor immunotherapy. Mice were immunized twice with tumor-associated peptide at barrier-disrupted skin and were challenged with B16 melanoma tumor cells. B16 tumor cells were virtually completely rejected after epitope peptide immunization via a disrupted barrier. Also, when tumor-bearing mice were treated with epitope peptide on tape-stripped skin, tumor cells regressed with peptide application, and 100% of the mice survived for one month and 95% for over 60 days. However, mice treated with peptide application to intact skin died after 34 days. Thus, topical immunization provides a simple, nonadjuvant system, and noninvasive means of inducing potent antitumor immunity that may be exploited for cancer immunotherapy in human.

TABLE 46.7
Using Tape Stripping in Understanding the Stratum Corneum

Subject	Description	Study
Method	Standardized tape stripping.	(2)
Tape stripping method was standardized and accompanied with advanced technique such as EPR and IR-D to identify SC quality and depth in the tape stripping process.		(71)
	Measurements by electron paramagnetic resonance spectra (EPR) correlated with that of visual score, TEWL, stratum corneum (SC) hydration and chromametry on surfactant-treated cadaver SC and stripped off SC.	(106)
	EPR is useful to access stripped-SC fluidity.	(106)
	Infrared densitometry (IR-D) provides accurate measurements of the SC depth for tape stripping.	(73)
	Quantification of SC by 96-well microplates.	(71)
	New lipidomic approaches, combined normal-phase liquid chromatography-electrospray ionization-mass spectrometry (NPLCESI-MS) with SC-stripped tape, was used in quantifying the overall ceramides (CERs) species in SC.	(80)
Percutaneous penetration/absorption	Tape stripping did not increase crotamiton absorption.	(99)
Different metals (copper, nickel) and new nanoscience technology (quantum dots) penetrating into skin were quantified by tape stripping.	Copper values, decreased from the superficial to the deeper layers in occlusive skin, were significantly above baseline in semi-occlusive skin.	(12)
	Occlusive application of nickel dust for 24 hours, the 20^{th} strip still indicated nickel present at 1.42 micrograms/ cm^2.	(100)
	Quantum dots penetrate human SC.	(101)
SC integrity	Lipid molecular organization is important for SC integrity.	(75)
Not only protein but also lipid components and related enzymes played important role in SC integrity detected by tape stripping[p].	Ceramide and ceramide synthases (CerS) are important in skin barrier function.	(82)
Skin damage and repair	Developing a TLR7- and TLR9-dependent skin inflammation via tape stripping.	(107)
Tape stripping can disrupt skin barriers and induce inflammatory reaction.	Epidermal IL-18 production associated with AD.	(89)
	Tape stripping increased skin TSLP levels which polarizes DCs to elicit a T(H)2 response via the induction of IL-10.	(108)
Changes of structure and mechanisms of disease skin were investigated by tape stripping.	6 hours after standardized tape stripping, epidermal mRNA of TNF-α, Hsp90, Hsp70, IL-33, and IL-8/CXCL8 upregulated, whereas CCL5/RANTES downregulated.	(109)
	Higher SC carbonylated protein level observed in psoriasis vulgaris and atopic dermatitis lesional area.	(90)
	Free amino acids and citrulline higher in non-occlusive than full-occlusive skin.	(76)
	Tape stripping via desmoglein 1 immunofluorescence in evaluating abnormal keratinization and healing process in response to treatment.	(110)
	Barrier recovery following tape stripping of the SC delayed in vitiligo.	(91)
	Tape-stripped wounds showed increased scar elevation index, epithelial thickness, and cellularity.	(111)
	Successive tape stripping caused a higher degree of barrier damage more rapidly in ostomy patients with peristomal skin problems.	(112)
	Immunofluorescent combined with tape-stripping used to measure activation-regulated chemokine in SC (scTARC), an indicator of the severity of local acute inflammation in patients with AD.	(113)
Nail	TOWL (transonychial water loss) increased in tape stripped nails, with no difference in removed protein quantity.	(88)
Tape tripping utilized on nail was developed.		
Bioequivalence	Bioequivalence of three marketed topical metronidazole evaluated by microdialysis sampling and tape stripping.	(105)
New cosmetics, topical drugs and techniques were compared with the traditional ones to standardize and certify its effects and functions.		
	Data from the pilot tape stripping study correlated well with that from the human skin-blanching assay.	(104)
Keratolytic	Similarly keratolysis with retinoic acid (RA), benzoyl peroxide (BP) and salicylic acid (SA) in 6 hours.	(114)
Evaluation of the effect, penetration rates, and sensitivities of keratolytics was simple and convenient via tape stripping	Reduced SC thickness, increased in baseline TEWL as well as a faster rate of increase in TEWL during tape stripping observed in skin treated with Aqueous Cream BP.	(115)
	Combined tape stripping with protein analysis sensitive in detecting keratolytic effect of salicylic acid.	(38)
Decontamination	Polypropylene glycol, polyglycol-based cleanser (PG–C) and corn oil more effective than traditional wash method.	(116)

TABLE 46.7
Using Tape Stripping in Understanding the Stratum Corneum (*Continued*)

Subject	Description	Study
By quantifying the materials inside SC after decontaminating treatment via tape stripping, more effective decontamination method was investigated.	Absorbing materials are better than washing.	(85)
Evaporation	No difference of evaporation between non-chamber device and closed chamber device in man in vivo.	(87)
Tested the skin evaporation in different situations.		
Dynamics	370 epidermal gene expressions varied at least once following SC removal by tape stripping.	(86)
Investigated the SC amount and genetic changes in tape stripping process to help further understand the tape stripping mechanisms.	Tapes utilized removed a similar amount of SC, but have a different propensity to cause barrier disruption.	(1)
Ethnic	Higher TEWL and lower pH found in black people after three or six tape strippings, but no	(117)
After tape stripping, skin of different ethnics showed different TEWL and PH values.	differences found in the deeper SC layers between ethnic groups.	
Extraction	Tape stripping and reverse iontophores have equivalent effect in qualifying amino acid in SC.	(77)
Tape stripping can extract drugs, topical drugs, chemicals, lipids and proteins penetrated SC.	2-pyrrolidone-5-carboxylicacid collected by tape stripping considered a biomarker of filaggrin genotype.	(78)
	Assessing the lipid peroxidation in SC and determining inhibition by topical antioxidants.	(79)
	Free ceramide concentration showed a higher concentration in deep layers, whereas protein-bound CERs remained constant.	(81)
Drugs Delivery	Lidocaine released from bioadhesive film to SC studied by tape stripping combined high-pressure liquid chromatography.	(94)
Drugs delivered from different formulations into SC were quantified and compared	Phosphatidylcholine/cholesterol liposomes promoted naproxen permeation.	(95)
	Microemulsion (0.1% w/v Nile red) consisting of (%, w/w) 15.4% oleic acid, 30.8% Tween 20, 30.8% Transcutol® and 23% water) interacted with the skin barrier via perturbing its architecture structure.	(97)
	Microemulsion components (oleic acid, Tween20 and Transcutol) penetrated into the SC in different extents.	(96)
	Topical application of naproxyl-dithranol (Nap-DTH) improved naproxen and dithranol delivery.	(98)
Enhancement technique	Iontophoresis enhanced deposition of dexamethasone sodium phosphate in stratum corneum and underlying skin.	(83)
Compare the chemical penetration with and without new enhancement techniques.		
Overview		(118)

Abbreviation: TEWL, transepidermal water loss.

UNANSWERED QUESTION AND CONCERNS TO TAPE STRIPPING

Surber et al. (70) reviewed the tape-stripping technique as standardized tape-stripping technique; many factors remain to be investigated. As shown in Table 46.2, the types and sizes of tapes utilized equally affect the method and the pressure applied to the strip prior to stripping. A proposed FDA guideline describes serial tape stripping to determine the amount of drug within the skin. By the guidelines, the first tape strip is discarded and the drug extracted from the remaining pooled strips and the quantified amount is expressed as a mass per unit area. From the guidelines, it is impossible to express the amount of drug substance per unit mass of SC and to determine the proportion of the SC that has been sampled by the tape-stripping method. Although tape stripping is relatively simple to execute, there are many opportunities for experimental artifacts to develop. Tape-stripping samples have a high surface-to-volume ratio, and losses by evaporation can be significant even for chemicals with relatively low volatility. In addition, the tape-stripping experiment is unsuitable for volatile chemicals (124). Considering the current application and convenient of tape-stripping method, topical vaccination and clinical trials for the determination of bioequivalence of topical dermatological products could be improved by stripping standardization.

USING TAPE STRIPPING IN UNDERSTANDING THE STRATUM CORNEUM

Tape-stripping methodology, as standardized by Löffler et al. (44) and Dickel et al. (71), has been widely used (72–74) and extended our knowledge of SC structure and function such as lipid molecular organization (75), amino acid (76,77), filaggrin (78), lipid peroxidation (79), and CERs (80,81). The important role of ceramide

synthases (CerS) in sustaining epidermal permeability barrier functions is also studied (82). Evaluation via tape stripping on new penetration enhancement techniques (83) and decontamination approaches (84,85) are recognized as reproducible, stable, and reliable. Further SC studies analyzing human skin dynamics (1,86), evaporation (87), integrity (75), nail transonychial water loss (88) and dermatologic diseases (89–93) are advanced with this technology. Its contribution in pharmacology in drug delivery (94–98), percutaneous penetration (12,98–100), keratolytics (102,103,38), and bioequivalence (104–105) is significant.

With this expanded but still far from complete knowledge, tape stripping not only separates the skin components but also facilitates the penetration of some chemicals and has the potential to promote dermatoxicologic and dermatopharmacologic assessment. Table 46.7 details some publications that emphasize the points.

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47 The diagnostic value of patch testing

Iris S. Ale and Howard I. Maibach

INTRODUCTION

Patch testing is a standardized diagnostic test routinely applied in clinical practice as the most important investigative method for studying delayed contact hypersensitivity (CHS). Properly performed and correctly interpreted patch testing represents the chief diagnostic method to confirm allergic contact dermatitis. (ACD) A detailed clinical history complements, but does not replace the patch test in diagnosing ACD. Properly interpreted, patch test reactions are acceptable as "scientific proof" of the cause of dermatitis. It may be considered as one of the most direct of all methods of medical testing, as it employs the agent that causes the disease; it applies that agent to the target organ, and it locally reproduces the pathogenic mechanisms and morphological changes of the disease itself. The procedure involves the epicutaneous application, under controlled conditions, of a specific substance (allergen) that should induce a cutaneous inflammatory reaction in the susceptible (sensitized) person; while causing no reaction in a non-sensitized person. The local reaction, reproducing the dermatitis "in miniature", provides a visible representation of the subject's general ability to react to the substance. Patch testing is very well tolerated, with very rare reports of systemic reactions.

The correct diagnosis of the allergens responsible for the patient's dermatitis, through a properly performed and interpreted patch testing, constitute the essential prerequisites for adequate therapeutic and preventive measures to be established. Patch testing results, used as a basis for subsequent avoidance, substantially improve the quality of life for dermatitis patients.

Even if extensively used in the diagnostic evaluation of ACD, patch testing is still underutilized in the evaluation of other immunoallergic disorders.

WHY AND WHEN PATCH TESTING?

Patch testing is indicated when an allergic component of the dermatitis is suspected. It has been shown to be significant both in confirming contact sensitivities suspected from the clinical history and in unveiling unsuspected sensitivities. A number of studies have shown that the history and physical examination alone is insufficient to consistently evaluate a patient with ACD (1–3). Cronin (1) studied 1000 patients by thorough clinical investigation and patch testing and demonstrated that the accuracy of the clinical prediction varied, depending on the characteristics of the clinical dermatitis and the causative allergen. For nickel, the most frequent sensitizer in women, the allergy was anticipated in 64% of the subjects, while chromate, the most common sensitizer in men, was suspected only in 40% of the cases. For other common allergens, such as lanolin and neomycin, sensitization was predicted in only 16% and 8% of the cases, respectively. Similarly, Fleming et al. (2) demonstrated that clinical questions were accurate to predict the causative allergen in only 29–54% of the ACD cases, depending on the involved allergen. Reliable identification of causative allergens, by history alone, represents an overwhelming task in which we are usually unsuccessful. Podmore et al. (3) patch tested 100 consecutive patients, 41 of them were tested for screening purposes (e.g., eczema without an obvious allergic contact factor or clinical contact dermatitis without an obvious allergen). In 59 patients, a contact allergen was strongly suspected. Diagnosis was confirmed in 32 patients. In addition, 17 patients had 23 unexpected positive reactions. At least 50% of the unexpected reactions were considered relevant to the patient's skin condition. If only the clinically suspected substances were tested, then all other possible sensitivities—which were not immediately evident from the history—would be neglected.

The major indication for patch testing is the investigation of probable ACD, constituting—together with a detailed clinical history and a complete physical examination—a crucial step in the diagnostic workup. The diagnosis of ACD involves: (*i*) Demonstrating the existence of delayed hypersensitivity to one or several allergens, (*ii*) demonstrating that the patient is exposed to the allergen(s) and, (*iii*) establishing that the hypersensitivity and the exposure explain the dermatitis under investigation (4). In this sense, a positive patch test reaction establishes that the subject has been previously exposed and sensitized to the allergen; nevertheless, it does not imply that the clinical exposure to the tested substance is the cause of the current dermatitis. The clinical history and/or additional provocative testing should determine whether there is a causal relationship between the alleged exposure and the clinical course of the dermatitis (5). Although patch testing is primarily conducted according to the clinical history and physical examination, the diagnostic process is bidirectional and the test results will direct further questioning and investigation. Reconsidering the history in the sight of the test results can lead to recognition of many hidden sources of causative exposure.

Patch testing should also be used to uncover contact allergy as a superimposed or complicating factor in endogenous or exogenous dermatitis other than ACD (6–8). Ideally, all patients with chronic or nonresponsive eczematous dermatitis should be considered for patch testing, especially those with hand—or hand and foot—dermatitis, which is clinically assumed to be irritant, dyshidrotic or hyperkeratotic; stasis dermatitis; genital and perianal dermatitis; numular eczema, and unclassified eczema (9,10). Patch testing might also be indicated when dealing with any dermatitis that persists for more than three months, is resistant to an appropriate therapy, or worsens during topical treatment. In many of these cases, the

offending agent is present in the topical products prescribed—or self-administered—for the treatment of the primary disease. Other patients in whom patch testing may be considered are those with, eczematous psoriasis, pustulosis palmaris et plantaris, essential pruritus, adverse drug reactions, or oral lichenoid reactions (11–14).

Finally, patch testing can be adapted for the study of several allergic disorders, including those mediated by immune mechanisms other than delayed hypersensitivity, for instance, contact urticaria or protein contact dermatitis; as well as diseases affecting organs other than the skin, such as, the respiratory or gastrointestinal tracts. (See the following).

THE VALIDITY OF DIAGNOSTIC PATCH TEST RESULTS

The validity of any test system is its intrinsic ability to detect or measure the aimed biological phenomenon, (i.e., to determine which individuals have the target disease and which do not), relying on the test capability to detect both true-positive and true-negative reactions, while minimizing the number of false-positive and false-negative reactions. In other words, can a positive patch test reaction predict contact sensitization with certainty or can it be elicited by a different biological phenomenon like contact irritancy or unspecific reactivity. A single patch test is a "snapshot" of the tempo of an evolving immunological process, and the issue of whether a positive patch test reaction is causally linked to the disease being studied involves several pitfalls including the inherent risk of false-positive responses, and the difficulties in assessing clinical relevance. As a bioassay, patch testing still confronts several inherent methodological problems, and requires strict observation of the technical aspects and critical assessment of the results. Recognizing the benefits of patch testing, as well as all its possible pitfalls, is of practical importance to the physician using this method for clinical diagnosis.

The ideal patch test should correctly diagnose contact sensitization, while producing no false-positive or false-negative reactions. However, even when an appropriate testing technique is applied, false-negative and false-positive reactions may occur. The frequency of false-negative reactions is difficult to evaluate. A false-negative reaction can occur for a number of reasons: (*i*) Failure to perform delayed readings, which is especially important for allergens known to elicit delayed reactions, and when testing elderly patients, who may present a protracted immunological response, (*ii*) the test concentration and/or the amount of the substance applied may have been insufficient, (*iii*) the vehicle may not have released a sufficient amount of the allergen (the biological availability was too low), (*iv*) the occlusion may have been insufficient, (*v*) the test site may have been inappropriate, (*vi*) the patient's skin may have been unresponsive due to prior sun exposure, local application of corticosteroids, systemic administration of corticosteroids or immunosuppressors or other causes of skin hyporeactivity, and (*vii*) there may have been an inadequate replication by the test of the real exposure conditions, namely concurrent occlusion, heat, mechanical trauma, or others, that may have enhanced the percutaneous penetration of the allergens and they could not be appropriately reproduced in patch testing. When patch testing with a particular substance is negative in a patient, who has an evident dermatitis from contact with that substance, the putative allergen should be retested—perhaps in a different concentration, with a different vehicle, or with a different testing method, such as the Repeated Open Application Test (ROAT). Likewise, the investigator must be aware of the pitfalls of false-positive reactions. A false-positive reaction may be attributed to several causes, such as: (*i*) Testing with allergens that are marginally irritants, (*ii*) testing with allergens at concentrations that exceed their irritancy thresholds, (*iii*) spillover reaction from a nearby true positive reaction, (*iv*) multiple simultaneous positive reactions, and (*v*) testing patients with active dermatitis or otherwise sensible or irritable skin. Certainly, these lists are not exhaustive.

The issues of skin hyper- and/or hyporeactivity would be better assessed if appropriate negative and positive controls were routinely applied in clinical patch testing. The negative control would be a chamber containing only petrolatum, while the positive control would be a mild irritant such as 75% aqueous propylene glycol, 20% nonanoic acid, or 0.25% sodium lauryl sulfate. To discriminate between false-positive reactions and true allergic reactions, we can repeat the patch testing of the individual allergen with lower concentrations or serial dilutions, or perform additional tests such as open or semi-open tests (15,16). Irritation reactions in the ROAT are very limited compared to patch tests.

Even if the allergic nature of a positive reaction, as read by international guidelines, cannot be taken for granted, for most common allergens a positive patch test reaction is predictive of contact sensitization. The validity of patch testing may be considered as good for many allergens, if tested under controlled conditions and in the proper concentration, and patch testing is performed and evaluated according to the international guidelines (17).

What are the Indicators for Evaluating the Validity of a Diagnostic Test?

The statistical principles that underlie the evaluation of diagnostic tests are frequently overlooked by clinicians. These principles are substantial in recognizing the inherent limitations that are present when applying diagnostic tests. We can consider the general criteria, which are the same as those used to assess the therapeutic interventions; namely, effectiveness, safety, acceptability, and costs. There are also criteria specific to the assessment of tests; they are, sensitivity, specificity, the relationship between sensitivity and specificity, reproducibility, predictive value, and likelihood ratio (LHR). These basic biostatistical concepts must be taken into consideration when validating a test as a diagnostic tool. These indicators compare the diagnostic discrimination of the test to the reference criterion or gold standard, which, by definition, has 100% sensitivity and specificity. The concept can be delineated using a 2 x 2 contingency table that takes into account the test result (i.e., interpreted as positive or negative) and the presence or absence of the disease being studied (Table 47.1). The key to patch testing is to allocate the tested individuals into either those who are allergic to the test chemical and should have a positive result or those who are not allergic and should have a negative result. Those instances in which the test results are positive, but the disease is not present, are called false-positive results. The negative test results found when the disease is actually present are called false-negative results. The proportion of subjects with a positive test result, out of all those with the disease is known as the sensitivity of the test. In our scenario, it measures the proportion of allergic individuals, who are correctly identified by the test; *ergo*, it measures how sensitive the test is to detect contact allergy.

TABLE 47.1
Statistical Indicators for the Validity of Patch Testing Results

Patch Test Result	Contact Allergy		Predictive Value
	Present	*Absent*	
Positive	True positive (TP)	False positive (FP)	Positive (TP/TP + FP)
Negative	False negative (FN)	True negative (TN)	Negative (TN/TN + FN)
	Sensitivity (TP/TP + FN)	Specificity (TN/TN + FP)	

Sensitivity = TP/(TP + FN).
Specificity = TN/(FP + TN).
Positive predictive value = TP/(TP + FP).
Negative predictive value = TN/(FN + TN).
Positive likelihood ratio (LR+) = sensitivity/(1 − specificity).
Negative likelihood ratio (LR–) = (1 − sensitivity)/specificity).
Prevalence = (TP + FN)/(TP + FP + FN + TN).
Pretest odds = prevalence/(1 − prevalence).
Post-test odds = pretest odds × likelihood ratio.
Post-test probability = post-test odds/(post-test odds + 1).

Specificity is the proportion of subjects without disease, with an appropriate negative test result. It measures the proportion of individuals without contact allergy, who are correctly identified by the test as non-allergic. In other words, sensitivity and specificity indicate the proportion of individuals who have been correctly identified as allergic or not allergic. These indices provide stable estimates of the test's diagnostic discrimination and can be applied to any diagnostic test, irrespective of the characteristics of the population on which the test is used (18–21). Although the concepts of sensitivity and specificity are required to determine the validity and accuracy of a diagnostic test, from a clinical point of view it is more important to determine to what extent the test can help estimate the probability of the presence or absence of disease, after testing. In other words, in clinical practice it is essential to know how a particular test result predicts the risk of disease. The percentage of true-positive results out of all the positive test results is referred to as the positive predictive value of the test (Table 47.1). It represents the probability that a patient with a positive test result actually has the disease. Similarly, the percentage of true-negative results out of all negative test results is referred to as the negative predictive value of the test. The positive and negative predictive values are of great importance for clinicians, who interpret the test results on a case by case basis. However, these values are influenced not only by the sensitivity and specificity of the test, but will also vary depending on the prevalence of the disease in the population upon which the test is applied. Thus, the significance of a test result is determined not only by the sensitivity and the specificity of the test itself, but also by the prevalence of the condition in the studied population. If the rate of contact allergy in the population tested is low, then the negative predictive value increases and the positive predictive value decreases. Conversely, when the rate of allergic persons tested increases; that is, patch testing is used mostly to confirm the clinical diagnosis; then the positive predictive value will increase at the same test sensitivity, while the negative predictive value will decrease (18–22). These statistical considerations have decisive clinical implications. In clinical testing, positive reactions are at least ten times less frequent than negative ones. Therefore, even assuming that the test has high specificity, the false-positive reactions will have a great impact on the proportion of the true positives out of all the positives elicited (i.e., the positive predictive value of the test). This substantiates the importance of achieving a high prevalence rate of truly sensitized patients through a careful clinical assessment before patch testing (21).

As the Predictive Values depend on the prevalence of the disease, they can rarely be generalized beyond the study (except when the study is based on a suitable random sample, as is sometimes the case for population screening studies). To overcome the difficulty, decision analysts have proposed an alternative method to assess the predictive properties of a test: the LHR (23,24). LHRs are alternative statistics for summarizing the test's diagnostic accuracy, which is especially helpful in clinical practice. Conceptually the LHR is the ratio of two probabilities, namely the probability that a specific test result is obtained in patients with the disease, divided by the probability of obtaining the same test result in patients without the disease. In the case of dichotomous test measures, the LHRs have a direct relationship with sensitivity and specificity that can be summarized as follows: Positive likelihood ratio (LHR+) = sensitivity/(1 – specificity); Negative likelihood ratio (LHR–) = (1 – sensitivity/specificity). A LHR greater than 1 indicates that the test result is associated with the presence of the disease, whereas, an LHR less than 1 indicates that the test result is associated with the absence of disease. An LHR of 1 implies that the test result is equally likely to occur among patients with the disease as in patients without the disease. The further LHR is from 1 the stronger the evidence for the presence or absence of the disease. LHR above 10 and below 0.1 are considered to provide strong evidence to rule in or rule out diagnoses, respectively, in most circumstances. LHR+ from 5 to 10 and LHR- from 0.1 to 0.2 provide moderate evidence for the presence or absence of disease. Tests with LHRs ranging from 0.33 to 3 rarely alter clinical decisions. LHRs are ratios of probabilities, and can be treated in the same way as risk ratios for the purposes of calculating confidence intervals (20,25). Theoretically, LHRs (unlike predictive values) are independent from the prevalence of the disease. Practically, LHRs may differ across various clinical settings and may be affected by the same limitations as predictive values. An alternative way to compare tests is by means of the diagnostic odds ratio. The diagnostic odds ratio is calculated as: (sensitivity × specificity)/[(1 –sensitivity) × (1 – specificity)] or as LHR+ divided by LHR – 0.20. Potentially useful tests tend to have diagnostic odds ratios well above 20 (e.g., an LHR+ of 7 and an LHR- of less than 0.3) (26).

To validate a test as a diagnostic tool, we must be able to discriminate how many times the test has accurately classified the tested subjects. Achieving this goal requires knowing in advance, which subjects had the disease being studied based on some reference test. The optimal design for assessing the accuracy of a diagnostic test is considered to be a prospective blind comparison of the test, and a reference test or gold standard in a consecutive series of patients from a relevant clinical population (26). As patch testing constitutes the only reliable and readily available test for diagnosis of contact allergy, the gold standard for comparison must be a confident clinical diagnosis made through the exhaustive study of each case and fulfillment of a precise case definition, in terms of clinical findings, history of exposure to the

tested substance, and reproducibility of the response, with an appropriate time course after exposure (27). Alternatively, an ROAT or controlled exposure to the tested substance can be envisaged as a reference for comparison (28). However, these tests also have a certain degree of ambiguity and need further standardization (29). Available data concerning validity of patch testing as a diagnostic tool are quite limited because, on clinical grounds, we usually do not apply diagnostic tests to groups of subjects who are known to have the disease we are trying to diagnose, (i.e., with incontrovertible contact sensitivity to the substances being tested). Similarly, data regarding testing in subjects without contact dermatitis are scarce. To assess the validity of patch test screening trays in the evaluation of patients with allergic contact dermatitis, Nethercott and Holness (30), tested 1032 patients, 639 of them with the International Contact Dermatitis Research Group (ICDRG) standard series and 393 with the North American Contact Dermatitis Group (NACDG) standard series, with the use of Al-Test patches or Finn Chambers. They found that the sensitivity, specificity, positive accuracy, negative accuracy, and validity index for the ICDRG and NACDG screening series were 0.68, 0.77, 0.66, 0.79, 0.72 and 0.77, 0.71, 0.66, 0.79, 0.74, respectively. Applying Nethercott's estimates for sensitivity and specificity, can be calculated, the LHR+: sensitivity/–1 – specificity) = 0•68/(1 – 0•77) = 2•95, and the LHR(: (1 - sensitivity)/specificity = (1 – 0•68)/0•77 = 0•43. Therefore, although both screening series scored relatively high, nearly 30% of all patch test results were considered inaccurate. Note, however, that the authors considered those patients with positive test results, in which investigation did not provide evidence to support clinical relevance (either present or past), as having false-positive tests. Similarly, patients with negative test results to the screening series, in which further testing revealed positive responses to other allergens, were considered to have false-negative screening tests. The issue of patch test validity is problematic, in that, patch testing does not represent a particular test, but rather a technique of testing. Thus, sensitivity, specificity, predictive values, or LHR data may be allergen-specific and will vary depending on the allergens tested and also according to the degree of severity of the patch test reaction. Thus, we have to take into account that the accuracy of the clinical patch testing may be higher for one allergen than for another, and also, higher in strong positive reactions versus weakly positive reactions. In addition, not only the allergen, but also the allergen concentration has to be considered when assessing patch test validity. The outcome of an individual patch test not only depends on the existence of delayed hypersensitivity to the tested substance, but also on the test concentration and the delivered dose, which will depend on the amount of percutaneous absorption induced by the method of exposure (31). Delayed sensitivity is a dose-related phenomenon and there is a threshold surface concentration of allergen required to induce sensitization and/or elicitation of the response (32–44). Therefore, the concentration of the allergen has an essential role in determining the amount of positive test results to be obtained. The dose of the allergen should be kept sufficiently high to detect allergy in weakly sensitized individuals, but low enough to minimize irritant reactions and the risk of sensitizing the patient. Almost any substance is capable of inducing irritant responses depending on the concentration and the method of exposure. When a test substance has low irritant properties, it is possible to use a relatively high elicitation threshold concentration; hence, allergic reactions will be more likely elicited. Conversely, if the substance has a fairly high irritancy potential, then a lower elicitation threshold concentration will have to be used to avoid the induction of false-positive irritant reactions. In the latter circumstance, allergic reactions are less likely to be elicited, especially in weakly sensitized persons. Variations in the cutoff concentrations will determine changes in the balance between positive and negative results (36–44). If the elicitation threshold concentration is raised, both the true-positive and false-positive test results will increase and the number of false negatives will decrease; the sensitivity increases and specificity decreases. Conversely, if the elicitation threshold concentration is reduced, we will have less false-positive test results, but also more false-negative responses. The specificity increases, but sensitivity declines. Therefore, the sensitivity and specificity of the test, as well as the predictive values are related to the elicitation concentration (Table 47.2). The choice of allergen dose is frequently a delicate compromise; it should maximize the possibilities of obtaining true-positive results, while minimizing the anticipated number of false-positive irritant results, in non-allergic subjects. Commonly, patch test concentrations for many allergens, even for allergens in the recommended standard screening trays, have been established in testing groups of patients supposed to have allergic contact dermatitis. In this context, a concentration is considered to be adequate when it is capable of eliciting a reasonable proportion of true-positive test results, (i.e., positive results, which are accepted to be in association with the contact allergy to the test substance, based on clinical grounds), while eliciting a reasonably low proportion of irritant results according to the morphological criteria. However, cutoff concentrations would be better estimated by employing the serial dilution test technique on patients proved to be sensitive to the tested substance, through controlled exposure, and also, on non-sensitive controls. Using this technique it would be possible to establish the concentrations eliciting strong, optimal, and minimal reactions. Thus, the mean standard error and ranges of reactivity for the different allergens can be calculated. Quantitative data about irritancy of the different substances can be obtained as well. This procedure has been used to standardize some patch testing materials, such as TRUE Test™. The cutoff concentration for TRUE Test allergens was determined as the minimum concentration that caused a 2+ reaction in at least 90% of the sensitive patients (45,46). The comparative multicenter studies with TRUE Test and Finn chamber technique indicate proximity in limits of weak sensitization and irritancy, with nickel, dichromate, cobalt, balsam of Peru, fragrance mix, carba mix, and thimerosal (47–50).

TABLE 47.2
Effect of Changes in the Elicitation Threshold Concentration

Elicitation Threshold Concentration ↑	Elicitation Threshold Concentration ↓
⇓	⇓
True Positives ↑ False positives ↑	True Positives ↓ False Positives ↓
True Negatives ↓ False Negatives ↓	True Negatives ↑ False Negatives ↑
⇓	⇓
Positive Predictive Value ↑	Positive Predictive Value ↓
Negative Predictive Value ↓	Negative Predictive Value ↑
Sensitivity ↑ Specificity ↓	Sensitivity ↓ Specificity ↑

The Reproducibility of Patch Test Results

The value of any test depends on its ability to yield the same result when reapplied to stable patients. Reproducibility of patch testing, defined as the test's ability to give consistent results when testing is repeatedly performed on the same individual, has been frequently questioned. Some authors have pointed out the low reproducibility of the patch test responses when testing was performed in duplicate on different body areas, such as the right and left sides of the back (51,52). Golhausen et al. (51) double tested concomitantly on the left and right sides of the upper back of 35 patients with allergens from the standard series and some vehicles (ointments), and found that 43.8% of the positive allergic reactions were non-reproducible. Subsequently, they reported a higher incidence of non-reproducibility of duplicate patch testing using allergens in petrolatum and Finn Chambers (37.9%) when compared with the TRUE Test (17.9%) (52). In a multicenter study from the German Contact Dermatitis Research Group, (53) 1285 patients were double-tested concomitantly with 10 allergens from the standard series and manually loaded patch test systems. Non-reproducible allergic reactions were seen in 194 patients (15.1%). The authors concluded that the non-reproducible results were highly dependent on the allergen tested. In contrast to these results, other authors (54–58), have reported that the reproducibility of patch testing was high. Lindelof (54), while testing 220 consecutive patients, obtained a non-reproducibility rate of 9.5%, Bousema (55) obtained 93% concordant allergic results, and Bourke et al. (56) reported 8% of completely discordant results, and observed that many non-reproducible results were not relevant. Using ready-to-apply systems, the reproducibility rate was even higher. Lachapelle (57) tested 100 consecutive patients, using Epiquick™ and observed a non-reproducibility rate of 4.2%, and Ale and Maibach, (58) using TRUE Test™, in 500 consecutive patients, obtained 95% concordant allergic results. Therefore, differences in the reproducibility of patch testing are possibly mostly due to the methodological aspects (58–60) (see next).

TABLE 47.3
Sources of Unreliability in Diagnostic Patch Testing

Materials
- Type of patch test system
- Different sources of patch test allergens
- Different vehicles and concentrations for some allergens
- Uneven distribution of allergens in the vehicle

Methodology
- Amount of allergen applied
- Regional variations in skin absorption and responsiveness
- Dissimilar pressure supported by the system according to the area of application
- Criteria of patient's selection
- Application and reading times
- Interpretation of the responses (intra-individual and inter-individual variability)

Technical
- Partial or complete detachment of patches
- Errors in the sequence of consecutive allergens

Biological
- Unresponsiveness (overlooked intercurrent factors such as sun exposure, drugs, etc.) Weak and doubtful responses
- Summation of individual responses
- Hyper-responsiveness and excited skin syndrome

ISSUES THAT MAY AFFECT THE VALIDITY OF DIAGNOSTIC PATCH TESTING

Issues Related to the Patch Test General Methodology

Conventional diagnostic patch testing has two main drawbacks: (*i*) It is very technique-dependent and (*ii*) nonspecific reactions are more common than with other skin test methods. Standardization of the patch test technique is essential for valid and reproducible results. Therefore, during the last decades of the twentieth century, great efforts were made to the optimization and standardization of patch sensitivity × specificity)/[(1 –sensitivity) × (1 – specificity)] testing materials and methodology. Significant research on the chemical and toxicological aspects of test allergens, appropriate vehicles, and skin penetration, have all contributed to the development of reliable and consistent patch test techniques. Yet, systematic studies for several important aspects are yet lacking. Several factors may influence patch test results and many sources of unreliability still exist, including variations in patch test materials, technique, and methodology, as well as, inherent biological variability of patch test responses (Table 47.3). The use of an appropriate vehicle is crucial. Vehicles influence the bioavailability and subsequent percutaneous penetration of allergens (61–70). Petrolatum remains the standard vehicle for most allergens, with the exception of TRUE Test™. However, the adequacy of petrolatum as a vehicle for many allergens has been questioned (65–70). Patch test suspensions in petrolatum contain undispersed allergen particles, and both the particle size and number differ significantly between different test substances and different manufacturers (65,66). This phenomenon was specially described for metal salt preparations (67–70), and the non-homogeneous release of allergens from the vehicle could result in false-positive reactions (69). Other test substances, such as disperse dyes (71–73), also produced a number of problems. Ryberg et al. (71) analyzed commercial patch test preparations of eight different disperse dyes from different suppliers and observed wide variations in concentration compared with the label, impurities, and even presence of a different dye allergen, in the final preparation. Frick et al. (72) performed a chemical analyses of 14 commercial test preparations of diphenylmethane-4,4'-diisocyanate in petrolatum and observed a poor correlation between the stated and found concentrations. Nowadays, efforts are being made to optimize the preparations and assure a homogeneous dispersion and stable concentration of the allergens. Skin absorption can vary greatly depending on the patch test system used (71). Factors such as conformity to the skin surface and degree of occlusion could be responsible for the differences in the kinetics of allergen penetration. Variations in the amount of material applied can also lead to erroneous results (74). Excessive amounts can provoke a spillover and irritant reactions, while inadequate dosing may, conversely, result in false-negative and doubtful reactions (75). The ideal test situation is a test area completely covered with the test preparation, without any spreading outside that area (76). The amount of material applied with the Finn Chamber technique should approximate 20 μl, (77) but, as a manually dispensed system, the amount of allergen applied is potentially variable depending on the technique (75,78,79). This

variation was reported to be higher when testing allergens in solution (80). Advances are being made in the optimization of patch test preparations and the dispersion of allergens, as well as the quality of these materials have significantly improved in the last fifteen years (81), but not much significant research has been done on alternate vehicles in patch testing (82). Problems associated with application seem to be solved with "ready-to-use" delivery systems, such as TRUE Test™, which has been pharmaceutically optimized concerning stability, solubility, and bioavailability of the allergens. In the TRUE Test system, the allergen is dissolved in an aqueous or ethanol solution and then incorporated in a dried-in-gel vehicle such as a polyvidone or a cellulose derivative. A thin layer of this gel is then coated onto a polyester sheet and dried to form a patch. The sheet is cut into $9 \times 9\,mm^2$ patches and arranged in panels, on strips of tape. The TRUE-test produces an exact dosage, even surface spread, and high bioavailability for the allergens. The allergen dosage has been determined by dose–response studies and the amount per unit area has been standardized (83,84), solving the problems of low bioavailability, uncertain dosage, and uneven surface distribution, which are commonly seen when petrolatum is used as the vehicle. However, only the standard series and other additional allergens are currently available with the TRUE Test.

Further methodological sources of unreliability are, the existence of regional and intra-regional variations (e.g., upper vs. lower back) of patch test responses (85–87) and miscellaneous individual factors, namely, menstrual cycle (88,89), seasonal variations (90), and so on. Finally, standardizing the application time is required to achieve comparable results (91–98). The test is generally kept in place for a period of 48 hours, although in some centers it is applied for only 24 hours.

Issues Related to Allergen Characterization and Chemical Variety

Ideally, allergens should be chemically defined and have high purity and stability. Standardized, commercially available allergens should be used whenever possible. Most allergens of the standard series are pure chemicals, such as nickel sulfate, cobalt chloride, formaldehyde, and so on. Others are chemically defined mixes of allergens such as the thiuram mix, mercaptomix, fragrance mix or paraben mix. Neomycin sulfate consists of three different chemical substances-neomicyn A, B, and neamine. Finally, some testing materials are complex natural products, such as Balsam of Peru (CAS 8007-00-9; 8016-42-0), colophony or wool alcohols. Much research is necessary to clarify the chemical structure of these natural materials and to define and characterize their allergenic fractions (99,100). In addition, some studies have found poor stability for some allergens (101–105). Allergenic degradation products can be formed during storage, mostly by oxidation, as in the case of terpenes, such as limonene and linalool (103). In these circumstances, it may be difficult to determine the real allergenic fraction. The optimal form of presentation is debated for certain allergens, even for well-defined ones. Nickel is a good example, for which sulfate is the present standard. Some authors, however, consider that chloride is more adequate (106). Gold and mercury present similar problems (107,108).

Additional problems were derived from testing with mixes of individual allergens. Mixes were used as screening tests, to depict the contact allergy to one or more of their constituents. They were designed as a simple method of increasing the number of chemicals tested, while decreasing the number of patches applied. Yet, the use of mixes involved problems of concentration, interference, stability, formulation, and validation (109–125). To avoid the occurrence of irritant reactions, the allergens were incorporated at suboptimal concentrations, sometimes resulting in false-negative reactions (109,111,117,118), The fragrance mix I, introduced as a screening tool in the late 1970s, following the study by Larsen (114), contained eight fragrance materials: eugenol, isoeugenol, oak moss, geraniol, hydroxycitronellal, α-amylcinnamic aldehyde, cinnamic aldehyde, and cinnamic alcohol. It also contained the emulsifier sorbitan sesquioleate, at 5% concentration, in order to achieve a satisfactory dispersion of the constituents in the petrolatum vehicle. It was considered that the fragrance mix detected 70 to 80% of the cases of fragrance sensitization (115,116). Not infrequently, the originally used formulation containing 2% of each constituent ($8 \times 2\%$) resulted in false-positive irritant reactions. Therefore, the concentration was lowered to 8 x 1%. However, the currently used concentration causes false-negative reactions (117,118) and still induces irritant reactions (119,124). There are also discrepancies between the patch testing results with fragrance mix and its constituents. A positive reaction to one or more of the fragrance mix constituents is seen in only 40 to 70% of the patients, with a positive reaction to the fragrance mix (122–124). The possible explanations for this discrepancy have been proposed in an excellent review by de Groot and Frosch (116), including:

1. False-positive (irritant) reactions to the mix.
2. False-negative reactions to the constituents. This may be due to: (a) cross-reactions between chemically related substances in the mix, (b) an additive suprathreshold effect of the individual constituent allergen, which may have suboptimal non-inflammatory concentrations in the mix, (126) (c) enhancement in the absorption of the mix constituents by the emulsifier sorbitan sesquioleate (SSO), and (d) a marginally irritant constituent of the mix may enhance the absorption of the other constituents.
3. Two or more constituents of the mix form a new allergen ("compound allergy") (127).

Enders et al. (124) consider that most negative reactions to the individual constituents, in patients with positive reactions to the mix, are due to the emulsifier SSO, which optimizes the diagnostic power of the mix. Therefore, they recommend the addition of 1% SSO, when testing with the individual ingredients of the fragrance mix. The value of adding SSO has also been investigated in a multicenter study of the European Environmental and Contact Dermatitis Research Group (EECDRG) (119) by testing patients with fragrance mix I, its eight constituents, with and without 1% SSO, and SSO itself. The test concentration for SSO was 20% in pet. according to the previous studies (128). Positive allergic reactions to SSO were observed in 0.7% of the patients. The authors recommended the addition of SSO to the standard series in order to adequately evaluate a positive reaction to the fragrance mix. Negative reactions to the mix with positive reactions to the ingredients also occur. De Groot et al. (118) tested 677 patients with the fragrance mix (8 x 1%) and its eight constituents. Sixty-one patients (9%) reacted to the mix and to one or more of the ingredients, while four

(0.6 of all patients and 6.2% of all fragrance-sensitive patients) reacted to one of the individual ingredients in the absence of a reaction to the mix—even upon retesting with serial dilutions—and therefore, were deemed to have false-negative reactions to the mix. Even if the proportion of false-negative results was low, given the high prevalence of fragrance allergy, the number of missed allergies with the currently used mix may attain clinical significance. Testing with the individual ingredients of the mix in those patients clinically suspected of having contact sensitivity—even when the reaction to the mix was negative—might contribute to solving this problem. The same consideration was valid when there was a suspicion of a false-positive reaction due to irritancy (117). As discussed above, the currently used fragrance mix I (8 x 1% with 5% SSO) is not perfect. It causes both false-positive and false-negative reactions and leaves 20–30% of the fragrance sensitivities undetected. With regard to the rubber mixtures, Geier and Gefeller (125) published a thorough study in 21.000 patients from the database of the Informational Network of Dermatological Clinics in Germany (IVDK). They focused their analysis on the reliability of patch testing with the mixes of rubber ingredients, as a marker for the detection of contact allergy to any of its constituents. The gold standard for comparison was breakdown patch testing, and the sensitivity of the mix was defined as the proportion of patients showing positive results to the mix, among the number reacting to any of its single constituents. The Thiuram-mix elicited positive reactions in 222 patients (9.8% of all tested patients). Of these, 162 (73%) reacted to one or more components and 60 (27%) did not react, hence, were considered as false positives. Of the patients negative to the mix, 32 (1.6%) reacted to one or more components, and were deemed to be false negatives. The biostatistics for the Thiuram-mix were as follows: sensitivity 84, specificity 97, positive predictive value 73, and negative predictive value 98. For the mercapto-mix the sensitivity was 57 and the specificity 99. The statistics for PPD black-rubber mix were: sensitivity 65 and specificity 99. Only 224 patients were tested with the Carba-mix. Positive reactions were found in 20 patients (8.9%) and 12 of them were considered as false positives. In addition, two of the 202 negative reactions (1%) were considered false negatives. The sensitivity was 80 and the specificity was 94. The authors concluded that the sensitivity and specificity of Thiuram-mix was acceptable. However, in case of an allergic reaction to the Thiuram-mix, they recommended breakdown testing, as only about one-half of the patients positive to the mix had positive reactions to one of the individual components. The Mercapto-mix had a low sensitivity, consequently its replacement by mercaptobenzothiazol was proposed. Similarly, they proposed the replacement of the PPD black rubber-mix by the IPPD mix. It was difficult to reach a conclusion for the carba-mix because of the small number of tested patients.

Issues Related to Patient Selection

Before performing patch tests, we should critically consider all the information about the clinical history and physical examination and generate precise pre-test probabilities of meeting the case definition for ACD (27). An accurate clinical history and a proper physical examination are the best diagnostic tests we ever have (129). As previously mentioned, to ensure a high positive predictive value when patch testing individual patients, it is crucial to maximize the proportion of patients who fulfill the case definition for ACD. In other words, the technique of patch testing is most effectively utilized as a confirming tool in those patients in whom a diagnosis of ACD was made, based on strict clinical criteria. Performing a patch test as a "last recourse" for managing refractory patients, who otherwise do not meet the clinical criteria for ACD, would not be expected to yield good results. In addition, to determine the sensitization rates in epidemiological studies, not only does the test system have to be well defined, but even more so, the test population. The sensitization rates in an insufficiently characterized test population can hardly reflect the number of clinically relevant sensitizations in the general population. Only data on the clinically relevant sensitizations from a uniformly selected and well-characterized test population are suitable for making inferences. The pattern of allergic contact sensitization in a population is influenced by individual factors, such as sex, age, presence of atopy, presence of diseased skin, as well as factors related to exposure, including the chemical structure of the allergen, concentration, climate, and industrialization (130). An unequal frequency of positive patch tests is to be expected among groups of patients who differ with respect to individual variables. Christophersen et al. (131) evaluated the influence of individual factors on the patch test results from consecutive patients in seven centers in Denmark during a six-month period. They concluded that the results could only be compared after stratification or multivariate analysis and proposed a logistic regression model for standardization of the presentation of the patch test results.

Issues Related to the Application of Multiple Patch Tests

With the premise of increasing the sensitivity of the patch test procedure and detecting as many clinically relevant allergic subjects as possible, it is common practice to employ arrays of many test substances, grouped as a test series, in the routine evaluation of patients with suspect ACD. If the cutoff concentration for each individual allergen in the series is settled at a 95% upper confidence limit, then, from a statistical viewpoint, each time we test 20 substances in a non-sensitized person, there may be a 100% chance of eliciting a false-positive result from one of the substances tested. If we set the upper confidence interval at 99%, that is, assuming a false-positive response rate of 1% for each substance, we still have a 20% possibility of eliciting a false-positive result each time we test 20 substances (19,21). As we consider the tray of substances as a single screening test, rather than an assemblage of individual substances, we are dealing with a confidence interval of 80%, well below the conventional 95% confidence interval used in other diagnostic tests. If we wish to use a 95% confidence interval for patch test screening and reduce the number of false-positive reactions, it would be necessary to lower the cut-off concentration of the individual test substances, which will simultaneously reduce the true-positive response rate. Alternatively, we can consider reducing the number of test substances in the standard screening series to the indispensable minimum, to diminish the risk of false-positive reactions. The above-mentioned concepts stress the significance of assessing the clinical relevance of the positive reactions. Critical revision of the clinical history and use of pertinent additional tests are needed to establish the validity of patch test results.

Diepgen and Coenraads (21) delineated another problem associated with testing multiple substances. When estimating the differences in sensitization rates between two groups of subjects (e.g., between males and females or between atopics and non-atopics), we frequently perform pairwise comparisons using chi-square

tests—one for each allergen tested—setting a p-value of 0.05 as statistically significant. In this circumstance, and for a series of only ten allergens, there is a random possibility, of over 40%, of finding, by chance, a statistically significant difference for at least one allergen between the two groups. Therefore, this procedure increases the probability of a false rejection of null hypothesis, concluding that there is a difference in the sensitization rate between the two groups, when there is in fact no difference.

Issues Related to Patch Testing with the Standard Series of Allergens

Most clinical cases of ACD are caused by a relatively small number of chemicals. Because of this, patients are tested with a group of 20 to 25 relevant chemicals grouped in a "standard series" as a primary screening procedure for the diagnosis of ACD. These series have been recommended by research groups (ICDRG, EECDRG, NACDG), with minor changes in the different countries, due to regional differences in exposure to sensitizing compounds. Their constitution is based on the statistics of allergens and they are periodically revised to adapt to changes in exposure, introduction of new environmental allergens, and information regarding irritation, active sensitization, and so on. (132). When testing with these substances, it is reasonable to assume that most of the obtained positive reactions can be ascribed to contact allergy. The usefulness of this screening series has been confirmed (24,133–137). It is generally believed that the standard series alone detects 70 to 80% of all contact allergies (133), but, in a multicenter study of 4824 consecutive patients from five European Contact Dermatitis Departments, the sensitivities detected by the standard series alone ranged from 37 to 73%, depending on the testing institution (134). Patients without a positive patch test to the European standard series and positive reactions only to additional allergens varied in frequency from 5 to 23%. Sherertz and Swartz (135) found that 36% positive reactions occurred to allergens in the NACDG standard series exclusively, and overall, 76% reacted to one standard allergen. Cohen et al. (136) tested 732 consecutive patients with the NACDG standard 20 allergens, the NACDG extended series, and other allergens if estimated necessary. Of these, 363 patients (50%) had positive patch test reactions. Only 23% of the patients had positive reactions to the standard series of 20 allergens alone, and 37% had a positive reaction to a standard series allergen and an additional supplementary allergen. Therefore, 60% of the patients had at least one positive standard reaction. Forty percent of the patients had positive reactions only to allergens in the supplementary group. Of the total cohort of 363 reactors, 221 patients (61%) were considered to have had clinically relevant positive patch test reactions. Only patients with current clinical relevance were included in this group. Those patients reacting only to a standard series of allergens had a high rate of clinical relevance (69%). However, they accounted for only 23% of all patients with positive reactions and 15.7% of patients with current clinically relevant reactions. The remaining 74% of the clinically relevant reactions were detected with the use of supplementary allergens alone or in conjunction with the standard series. In a multicenter study from the Informational Network of Dermatological Clinics (IVDK) in Germany (137), 4140 patients were tested with the German Standard Series. Contact sensitization was diagnosed in 47% of the patients tested, varying between 30 and 64% in the different centers. Forty percent of all the patients, who proved to have contact allergy, were diagnosed by using the standard series alone, while 20% of the cases were diagnosed solely with additional allergens (occupation-specific or material brought in by the patient). Veien et al. (138) tested 6759 patients with the European standard series over a five-year period. Additional allergens were tested in 1450 of these patients. Positive reactions to the allergens in the standard series were seen in 1941 patients, while 236 of the 1450 patients (16%) also tested with substances not included in the standard series, and had one or more positive reactions. Of the 1941 patients with positive patch tests, 1705 (88%) reacted only to substances in the standard series, 98 patients (5%) reacted both to substances in the standard series and to additional non-standard substances, while 138 (7%) reacted only to non-standard substances. These variations could be attributed to differences in the population tested, (i.e., differences in exposure, in the number of additional substances tested, etc.). The absolute frequency of contact allergy in any population will never be known, but the more the substances and products are tested, the greater will be the observed percentage of sensitization.

Issues Related to Patch Testing with Non–Standard Allergens

Most patients should be tested to a standard series, additional series or individual allergens will be selected depending on the history and distribution of the dermatitis, and the occupation and geographic area. The characterization of a patch test allergen requires an adequate knowledge of its sensitizing capacity, its occurrence in the environment, and if possible, the results of testing a large number of subjects. Most allergens from the standard tray and the most commonly used "aimed" trays are chemically defined materials of high purity and a large amount of clinical data has been accumulated concerning patch testing concentrations. When testing with these substances, it is reasonable to assume that most of the obtained positive reactions have significance and that the investigation to assess its relevance is warranted. In contrast, testing with non-standard allergens, other than those of the recommended aimed trays should be undertaken with caution. These may be chemically pure substances, but often they are compound products and may contain unknown components. Some components can even be irritants; such is the case for many industrial products. Specialized textbooks regarding a test's concentration for many non-standardized materials are currently available (139). This information is of practical value as a starting point when testing with these materials. However, remember that for several of these substances there has been little research concerning test concentrations and suitable vehicles. When testing chemicals for which there is limited data, we are faced with the same problems with regard to the sensitivity and specificity of the test; besides, we have to determine the appropriate strategy of testing and the valid test concentration, and we lack the information to categorically substantiate any conclusion. Therefore, patch testing with those materials may have little to contribute diagnostically, unless there is a definite clinical suggestion of their responsibility in the causation of the dermatitis. Finally, there are substances for which no information exists, except the chemical composition, which is provided through the Material Safety Data Sheets for industrial products or the list of ingredients for household, cosmetic, and toiletry products. Usually these products are technical grade chemicals; therefore, it should always be considered that they may contain unknown components. Should any

substance be considered potentially irritant, an open-use test may be envisaged. It should be performed with diluted substances, whose concentration can be progressively increased as far as no response, either allergic or irritant, appears. It is often helpful to patch test an uncommon substance at two or three ten-fold serial dilutions, such as 1.0%, 0.1%, and 0.01%. This procedure will prevent seriously irritant reactions and may help to distinguish between irritant and allergic reactions. If an allergen is serially diluted, a gradual reduction in the intensity of the reaction usually occurs, while an irritant reaction will tend to disappear abruptly. A gradually declining reaction, still distinctly positive at 0.01% or even 0.001%, constitutes highly suggestive evidence of contact allergy to the tested substance. It has been the usual practice to determine the "safe" or "non-irritating" test concentration for new materials, by testing in control groups. Before any human test is performed, complete toxicological information of the material must be procured. In addition, the control subjects should be followed up for one month to rule out active sensitization. It is recommended to test at least twenty control subjects based on the premise that if none reacts at the selected concentration, then, the testing is above the 95% one-tailed confidence limit. Usually, the test is performed with several concentrations, selecting the highest non-irritant concentration as the elicitation (cutoff) concentration. The highest non-irritant concentration is then applied to the subject with suspect ACD to the substance. The development of a positive patch test response with the morphological attributes of an allergic reaction will constitute evidence of contact sensitization due to the substance.

Issues Related to the Reading and the Interpretation of Patch Test Responses

Even when the proposals of the International Contact Dermatitis Research Group in 1970, concerning a uniform terminology for patch test reading, were generally accepted and represented a great advance (Table 47.3) (140), reading of patch test responses needs to be considered as eminently subjective and constitutes one of the limitations of the method (141). Patch testing is a perceptual test, based on inspection and palpation of the test area. As any test that involves human perception and judgment, patch testing is bedeviled by the variability of reporting on results. There are two forms of variability: (*i*) Intra-observer variability, that is, the phenomenon in which the same observer classifies the same test result differently on two separate occasions and (*ii*) Inter-observer variability, that is, the phenomenon in which different observers classify the same test result differently. In epidemiological studies, this variability is recognized as an inevitable consequence of the use of perceptual tests.

The classification and score grading of patch test reactions depends on the descriptive morphology. The typical morphological features of an allergic test response are erythema, edema, papules, and vesicles (or bullae). The significant point in assessing a positive patch test response is ascertaining whether it represents an allergic reaction or a false-positive reaction. At least an erythematous infiltration should be present for a reaction to be considered allergic, while reactions that show only erythema without infiltration—called doubtful reactions—are frequently nonspecific or correspond to irritancy (140). Allergic patch test reactions are traditionally scored in terms of intensity, and a grading scale from 1+ to 3+ is now generally accepted for ranking these allergic reactions. Even when all reading systems are based on the same morphological features, there remains some variation in the exact definition of the different grades of this scale, between the different working groups. For instance, there are discrepancies in the reading of the 1+ reaction between different contact dermatitis groups. Some groups define the 1+ reaction as homogeneous redness in the whole test area with scattered papules, while others only require redness and homogeneous infiltration in the whole test area. Menné and White (141) have proposed to introduce an extra grade of patch test reaction in the scoring: (+) Homogeneous redness in the test area with scattered papules, (++) homogeneous redness and homogeneous infiltration in the test area, (+++) homogeneous redness and infiltration with vesicles, and (++++) homogeneous redness and infiltration with coalescing vesicles. However, it is debatable whether this distinction can have any practical benefit. In contrast, other authors have suggested that a simplified score may reduce the interindividual variations in patch test readings (142). No real consensus has been reached in this matter so far. Such minor differences of categorization may determine variations in interpretation of the responses (143). Bruze et al. (143) have studied the accordance in patch test readings and have shown that there is good accordance among various readers, except with the NACDG system. The morphological feature that seems most difficult to evaluate is the papule, so, perhaps it will be convenient not to demand the existence of this feature as essential for the categorization of a patch test reaction as allergic.

The time of reading has been standardized (144), but is somewhat variable between different patch test clinics. Usually, the first reading is performed at day two (48 hours) after the patch test application, approximately 30 minutes after taking off the patches, and the second reading is performed at 72 or 96 hours. Unfortunately, the timing of the reactions to different allergens does not necessarily follow the timing of the readings. Delayed readings, one week after application are highly recommended, especially for some slow-reacting allergens, such as neomycin or corticosteroids, among others; even nickel may be a slow reacting allergen (144–147). Patch test results should be read at least in two successive opportunities, without which, their accuracy is seriously impaired. A single reading on day two (48 hours) may determine that approximately 30% of the contact allergies detected by the standard series are missed, as compared with the number of allergies found when the test is read repeatedly up until one week from patch test application (144). In addition, multiple readings are crucial in distinguishing false-positive reactions. However, if only one reading is feasible, it should be performed on day three or four (144).

Doubtful(?) and weak(+) reactions require a cautious interpretation and a careful consideration of the clinical circumstance. When a weak reaction correlates with the clinical picture, it may be significant (148). On account of biological or technical reasons, there may be a variation in the intensity of the test response to the same allergen from time to time. To establish or rule out contact allergy, merely repeating the patch test may be sufficient to demonstrate that a doubtful or weak reaction is not consistently obtainable, and therefore, probably represents a false-positive reaction. If required, the patch test concentration may be raised and/or additional tests such as intradermal testing or provocative testing may be performed.

The ideal patch test should indicate contact sensitization and produce no false-positive or false-negative reactions. The background of false-positive test reactions is usually irritancy. Although

the recommended test concentration for the sensitizers in the standard series is the result of extensive international experience on testing, some of the concentrations (e.g., for chromate and formaldehyde) have been chosen too close to the irritancy threshold, in order to diminish the risk of obtaining false-negative reactions. It has been claimed that false-positive irritant reactions do not represent a practical problem to the experienced physician. However, irritant reactions are often morphologically indistinguishable from allergic reactions (149). Likewise, weak allergic reactions can also be clinically indistinguishable from false-positive allergic reactions. The distinction is not necessarily provided by conventional histology, nor yet appropriately resolved by specialized immunological (150–152) or bioenginnering techniques (153–156). Multiple positive patch test reactions should arouse the physician's suspicion to the Excited Skin Syndrome (157–159). The open-use or provocative test may sometimes distinguish an allergic from an irritant response, because open testing is far less likely than closed testing, to produce an irritant reaction. Ideally, the ambivalent patch test should be repeated, and possibly a dose-response assessment (serial dilution) incorporated (160).

The frequency of false-negative reactions is difficult to evaluate. Even with the appropriate patch test material there may be several reasons for false negativity, most often insufficient penetration of the allergen. Along with the intrinsic sensitizing properties of the substance, we consider the concomitant exposure factors that might enhance percutaneous penetration; that is, irritation, occlusion, heat, mechanical trauma, and so on. (161,162). These factors cannot be reproduced in patch testing. Skin hyporeactivity is a poorly investigated phenomenon that has to be considered as a possible cause of false-negative reactions (163).

ASSESSMENT OF CLINICAL RELEVANCE

Patch testing results require biological and clinical interpretation. The fact that contact allergy to a certain allergen(s) has been reliably demonstrated by careful patch testing does not prove that such an allergen(s) is responsible for the patient's ACD. A true positive patch test reaction only indicates that the patient has been previously exposed and sensitized to the substance. Patients may suffer major changes in their lifestyle on the basis of patch testing results; therefore, it is crucial to establish that the positive reaction is actually linked to the clinical dermatitis, either as a primary cause or as an aggravating factor. Assessing the relevance of a positive patch test reaction is complex and involves many confounding factors. Based on the presence of a putative allergen in materials that comes in contact with the skin, either occupationally or during leisure activities, the pattern of distribution of the skin lesions, the effect of elicitation by exposure, and healing of the dermatitis by avoidance, the positive patch test results are judged as possible, probable, or certainly relevant (164). According to the ICDRG criteria (165) we consider that a positive patch test reaction is "relevant" if the allergen is traced. If the source of a positive patch test is not traced, we consider it as an "unexplained positive". We use "current" or "present" relevance if the positive patch test putatively explains the patient's present dermatitis. Likewise, when the positive patch test explains a past clinical disease, not directly related to the current symptoms, we refer to that as "past" relevance. However, a recurrent, but discontinuous, contact with an allergen can occur in some patients, providing difficulty in discriminating between the current and past relevance. From a practical perspective, establishing a positive reaction has past relevance or a possible relevance does not direct the clinician to intervene directly for the very problem for which past testing was performed. However, reporting the not currently relevant data plays an important epidemiological role and may be useful in preventing further outbreaks of ACD in a patient.

The determination of relevance primarily depends on the expertise of the investigator and the possibility of detecting the allergen in the environment of the patient. In many cases, a positive reaction is judged as irrelevant owing to insufficient environmental information. Often, a visit to the patient's workplace proves rewarding. We must perform a rigorous environmental evaluation, investigating the existence of allergenic exposures, characteristics of this exposure, and the possible concurrent factors. Absolute proof of relevance is often unattainable, as it is frequently not known whether the suspected products actually contain the implicated allergen in a sufficient amount to elicit the dermatitis. Guidelines for assessment of relevance have been proposed (5) (Table 47.4). The relevance scores

TABLE 47.4
Suggested Guidelines for the Assessment of Relevance

1. Perform a thorough and standardized history (including sex, age, occupation, occupational and domestic exposures, adverse reactions, time course, effect of elicitation of the dermatitis by exposure and healing by avoidance)
2. Perform a meticulous physical examination
   Consider the primary site of dermatitis and the pattern of distribution of the skin lesions
   Look for clinical clues of specific exposure and the possible correlation with products containing the allergen in question
3. Determine the existence of allergenic exposure
   Qualitative exposure assessment (search for the presence of the responsible allergen—or a cross-reacting substance—in the patient's occupational or domestic environment)
   Product information (product labeling, material safety data sheets, information from manufacturers or suppliers, textbooks, and product databases)
   Chemical analysis of suspected products
   Quantitative exposure assessment
   Removal techniques, such as skin washing and wiping
   Surrogate skin techniques, where a chemical collection medium is placed on the skin
   Fluorescent tracer techniques
   Biological monitoring
4. Assess all exposure parameters
   Consider all possible types of exposure: direct, indirect, sporadic, and concealed
   Consider the possible route of exposure
   Evaluate the specific site(s) of contact and the presence of skin damage or previous dermatitis in the contact area(s)
   Determine the concentration of the substance in the suspected product
   Determine the intensity of exposure (i.e., dose, duration, frequency, and total surface area)
   Determine the existence of simultaneous exposure factors: humidity, occlusion, temperature, and mechanical trauma
5. Repeat the patch test and/or perform additional testing procedures
   Test the suspected allergen(s) with a different concentration (including serial dilution), vehicle, occlusion time or other variables
   Test with products brought by the patient presumably containing the suspected allergen and/or product extracts
   Perform repeated open application test, provocative use test, open or semi-open tests

Source: From Ref. 4.

and accuracy of the assessment are significantly improved by a comprehensive knowledge of the patient's chemical environment. Sometimes it is difficult to substantiate the presence of the allergen in the patient's environment. This may be due to the complexity in detecting certain allergens or insufficient knowledge about the composition of different products. As a consequence, the relevance scores for different allergens vary; the ease of identification of the source of an allergen gives higher relevance scores (5,165–167).

Besides patch testing, other types of skin tests, such as open and semi-open tests, tests with product extracts, or the ROAT, may be required to establish a definite causative relationship between the positive patch test result and clinical dermatitis. The ROAT has a significant potential in refinement of the evidence-based diagnosis of clinical relevance. This test is not standardized to the same extent and it is time-consuming, but mimics some real-life exposure situations. However, for general validation, a standardized measurement of the results of ROAT, such as the ICDRG scoring system for patch testing, is required (28).

An important effect seen in clinical practice is the differential verification of positive and negative results. As relevance is not assessed for negative reactions, we fail to identify the false-negative test results. Moreover, doubtful reactions may be clinically relevant according to undeniable clinical criteria or follow-up testing. It could be worthwhile to ascertain whether doubtful (?) or weak (+) patch test reactions yield a significantly different relevance score than stronger and presumably more reliable positive patch test reactions.

EXTENDED APPLICATIONS OF DIAGNOSTIC PATCH TESTING

Diagnostic patch testing is customarily applied for the investigation of ACD, which is a type IV delayed, T-cell mediated hypersensitivity to contact allergens (contact hypersensitivity reactions, CHR). However, it may be adopted to detect a full range of immunological reactions. In addition, patch testing can detect a wide variety of sensitivities to inorganic and organic chemicals, drugs, biological molecules, inhalants, and food allergens. Patch testing was exclusively used for detecting contact sensitivity until 1982, when Mitchell et al. (168) documented its value for diagnosis of mite sensitivity in eczema patients. In 1996, Isolauri and Turjanmaa (169) used patch tests with food allergens to detect food allergies that were not detected by prick testing. At the present time, patch testing is an optional modality for the evaluation of allergic disorders affecting organs other than the skin, such as gastrointestinal allergies, allergic rhinitis, and asthma.

Atopy Patch Tests

The atopy patch test (APT) involves the epicutaneous application of protein allergens in a diagnostic patch test setting. It has been introduced as a diagnostic tool for identifying protein allergens (mainly aeroallergens and food allergens) causing or exacerbating the atopic eczema/dermatitis syndrome (AEDS) (170). Recent data show that the APT may be positive also in patients with food allergies and respiratory allergies.

The APT has been recognized as a useful diagnostic tool in the diagnosis of a delayed type of reaction in AEDS, targeting the cellular component of the immunological response (T lymphocyte–mediated, allergen-specific immune response) (171). As a specific IgE (sIgE) and skin prick test (SPT) can be correlated with early reactions, they can be used together with the APT, as complementary tools, for studying the immunological spectrum of AEDS. In fact, the denomination atopy patch test seems inappropriate, because the test does not reveal atopy, that is, a type I hypersensitivity, but rather a subtype of delayed type IV hypersensitivity (171). Biopsy specimens of the APT sites in patients with AEDS are found to have an initial Th2 cell infiltration (172), followed by a predominant Th1 cell infiltration. Similar biopsy findings have been observed in the skin of atopic dermatitis patients having acute and chronic lesions (173). The close microscopic and macroscopic similarities between the specimens from the APT sites and the lesional skin of patients with AEDS indicate that the APT is a valid model to study allergic inflammation in AEDS (173). Moreover, the APT could be used in children with gastrointestinal reactions to foods as well as eczema (174,175).

As there is no gold standard provocation test protocol to confirm the relevance of airborne allergens as a cause of AEDS, the sensitivity, specificity, and predictive values of the APT with aeroallergens are very difficult to assess, and must be related to the objective clinical symptoms. Oral provocation challenge test remains the "gold standard" for the food allergy diagnosis. The provocation is ideally performed as a double-blind, placebo-controlled, food challenge (DBPCFC), for example, with "masked" (lyophilized) foods, in color and flavor neutral formulas, after at least two weeks of a corresponding elimination diet (176). Unfortunately, food challenge protocols need further standardization, especially for delayed food reactions. A European position article has been published on the use of provocation tests in the evaluation of immediate reactions to foods, but there is no agreement in the methodology of the tests for assessing delayed-type food reactions (177). In addition, false-negative food challenges have been documented to occur in up to 3% of the cases; therefore, all double-blind food challenges should be followed with open feeding of the food in its natural form (178). As the DBPCFC is difficult to perform in a clinical setting, single-blind and open food challenges may be used instead, when practicality must be taken into consideration. For AEDS, early (within 1–2 hours after provocation) and late reactions (2–24 hours) can be distinguished, therefore, diagnostic tests should be performed to investigate both immediate and delayed hypersensitivity reactions (169,171,179,180).

Isolauri E, Turjanmaa (169) reported a comparison between SPT and APT in children with AEDS and milk allergy. They noted that immediate reactions to challenge were associated with more positive SPT, whereas, delayed reactions were associated with more positive APT. Therefore, they suggested that a combined use of SPT and APT might enhance the identification of the food responsible for the allergy in these children. (181). Keskin et al. studied children with various symptoms of milk allergy, and observed that the positive predictive value of APT was higher than that of SPT for both immediate (87% vs 73%) and late reactions (60% vs 22%) (181). The combination of positive APT together with defined levels of specific IgE or SPT, appeared to make DBPCFC superfluous in some cases. In a multicenter European study, (182) it was demonstrated that the APT had a higher specificity than SPT, or specific serum IgE, in the investigation of aeroallergens and food allergens in atopic subjects. However, in a study on children with suspected cow's milk and egg allergy, the APT did not identify any responsible food that was not identified by SPT (183). Breuer et al. (184) analyzing, retrospectively, 106 DBPCFC to cow's milk, hen's egg, wheat, and soy in 64 children with AEDS, showed a poor reliability for the APT. These controversial data might be explained

by the fact that non-reactions occur frequently, making difficult the interpretation of the test. Apparently, a number of minor test modifications may have a significant influence on the sensitivity, specificity, and reproducibility of APT.

Rancé (185) studied four food allergens (hen's egg, cow's milk, wheat, peanut) in 48 children with AEDS, aged between three and twenty-nine months (median 14 months), using two different occlusion times (48 and 24 hours). Sixty-four open food challenges were performed and the sensitivity and specificity numbers were correlated with the positive challenge tests. For the 48-hour occlusion time, the sensitivity was 0.98, specificity 0.90, positive predictive value 0.87, and negative predictive value 0.92. The values were much lower for the 24-hour occlusion time. Niggemann et al. (186) found that the sensitivity, specificity, and predictive values improved when using the 12-mm chamber compared to the 6-mm diameter, and Perackis et al. (187) found that there was no difference between the age of children undergoing APT and its predictive value.

Concerning the reproducibility of APT, 16 patients with a history of AEDS and a history of positive APT reactions were studied by Weissenbacher et al. (188). The allergen was reapplied at the same time on both forearms and the back. The APT reactions were highly reproducible and positive in 15 of 16 (94%) patients. A reaction was more frequently positive on the back (94%) than on the arms (69%). Holm et al. (189) also found a relatively good reproducibility over time: Ten out of thirteen patients showed positive results by reapplication of the same allergen. The methodology and materials for APT were standardized by the European Task Force on Atopic Dermatitis (ETFAD) (Table 47.5) (190). It consisted of purified allergen preparations in petrolatum, applied in 12-mm diameter Finn chambers mounted on Scanpor tape to non-irritated, non-abraded, or tape-stripped skin of the upper back. Commercial protein extracts, such as food extracts, for skin testing have yet to be fully standardized and thus may have varying concentrations of relevant proteins. Therefore, fresh native foods or dried foods dissolved in saline or water can be occasionally used for APT. The APT is read at 48 and 72 hours according to the test criteria, with reading the key of the ETFAD for appearance of erythema and the number and distribution pattern of the papules (Table 47.6) (190).

Side effects of APT are uncommon, irritant reactions have been described (191). Contact urticaria has also been reported, although it may represent a concomitant immediate reaction (192).

Patch Testing in Food Allergies

The prevalence of food allergy is difficult to determine, as the reports vary according to the definitions used and populations studied. However, it is undeniable that food allergy is common and its prevalence seems to be increasing. In a recent meta-analysis, the prevalence of food allergy, diagnosed by objective tests, ranged from 1 to 10.8% (193). The last report by the Centers for Disease Control and Prevention estimates the prevalence of food allergy to be 3.9% in children younger than 18 years of age, in the United States, representing an increase of 18% from 1997 to 2007 (194). A recent National Institutes of Health-sponsored systematic review of food allergy concluded that food allergies affect more than 1 to 2%, but less than 10% of the U.S. population (195). As mentioned above, food allergy has been demonstrated to play an important role in the pathogenesis of AEDS. A relevant clinical hypersensitivity to food has been demonstrated in 30–70% of the patients with mild-to-severe AEDS in double-blind, placebo-controlled oral food challenges (196,197).

Testing for food allergies is not straightforward, as adverse reactions to foods may have a non-immunological nature, and those that are immune-mediated, may respond to different mechanisms, and sometimes to more than one immunological mechanism. Therefore, simple tests such as the SPT and serum food-specific IgE testing, which are the most commonly used diagnostic tests to evaluate for IgE-mediated food reactions, may be negative in true allergic reactions not mediated by food-specific IgE (198–200). Patch testing (APT) may be helpful in identifying late reactions to food in atopic dermatitis (as seen earlier) (194–200)and also in non-IgE-mediated, food-induced, gastrointestinal symptoms, such as those seen in eosinophilic esophagitis (201) and the food protein–induced enterocolitis syndrome. (202) In the study of Isolauri and Turjanmmaa, (169) 61 of 183 children with atopic dermatitis had a concomitant gastrointestinal reaction. They found that more than 80% of the patients with delayed reactions of vomiting and diarrhea after a food challenge had a positive patch test to a food antigen, whereas, 25% had a positive SPT. This suggested that patch testing might be used to detect gastrointestinal food allergies. Spergel and Brown-Whitehorn (203) studied 26 patients with eosinophilic esophagitis, confirmed on biopsy, with SPT and patch testing, and 75% of them had a positive test result to one or more foods. Removal of the causal food led to the resolution of symptoms. In addition, the eosinophils in the biopsy

TABLE 47.5
The European Task Force on Atopic Dermatitis (ETFAD) Protocol for APT

Test area: Uninvolved skin of upper back
No tape-stripping, scalpel abrasion or pre-treatment
Large Finn Chambers (12 mm) on Scanpor tape
Purified allergen in petrolatum as test substance with standardized allergen concentration (in biological units or µg/mL major allergen content)[a]
Occlusion time of 48 hours
Reading at 48 hours (20 minutes after removing of the set) and 72 hours
Exclusion criteria for APT:
• Test site free of topical steroids for seven days
• Test site without ultraviolet treatment for four weeks
• Patients free of oral steroids, cyclosporine A, or tacrolimus
• Avoidance of antihistamines for five days
• Non-pregnant

[a]Although the availability of standardized food allergens is poor and many foods contain more than one protein that can cause allergic reaction, fresh native foods or dried foods dissolved in saline or water can be used for APT (180).

TABLE 47.6
Key for Atopy Patch Test Reading (180)

?	Negative only Erythema	Negative Questionable
+	Erythema, infiltration	positive
++	erythema, few papules (< 3)	
+++	erythema, many or spreading papules (> 4)	
++++	erythema, papules and vesicles	

samples returned to normal levels. De Boissieu et al. (204) investigated 35 infants with possible cow's milk allergy, to assess the possible role of patch testing, in addition to the conventional SPT. They concluded that patch testing was helpful in the detection of cow's milk allergy with a sensitivity of 79% and a specificity of 91%. However, further standardization is needed before patch testing can be routinely applied in the diagnosis of food allergies in clinical practice (205).

Patch Testing in Cutaneous Adverse Drug Reactions

In order to appreciate whether skin testing may be useful in elucidating causality in drug allergy, it is vital to understand which type of immunological reaction is detected by which type of skin test. Patch testing with a suspected drug has been reported to be helpful in determining the cause of cutaneous adverse drug reactions (CADR) mediated by Type IV delayed hypersensitivy mechanisms (206–208).

It should be taken into account that type IV hypersensitivity to drugs can manifest in a great range of clinical patterns, with a confusing diversity of names, including maculopapular rashes, toxic erythemas, urticaria-like eruptions, acute generalized pustular eruption (AGEP), fixed drug eruption, drug reaction with eosinophilia and systemic symptoms (DRESS), erythema multiforme, Stevens Johnson syndrome (SJS), and toxic epidermal necrolysis (TEN). The use of terms such as urticaria-like, for example, although common in clinical settings, has a major risk of confusing type I- and type IV-mediated processes (209).

The aim of patch test investigation in a patient with a drug eruption is to identify the incriminated drug in a safe and relatively easy way. There are few reports on serious immediate reactions elicited by patch testing (210). However, severe CADRs generally constitute a contraindication for cutaneous testing, and, if testing is performed, it should be done with extreme caution (211).

Some key issues about patch testing with drugs must be taken into account, including the formulation, the site for testing, which clinical patterns are associated with, positive patch tests, and which drugs can elicit positive patch tests. Drug patch tests are performed according to the methods used in the study of ACD. Patch test reactions need to be read at 20 minutes, day two, and day four. Whenever possible, if the patch tests are negative on day four, a reading should be performed on day seven. Results of patch testing should be reported according to the International Contact Dermatitis Research Group (ICDRG) criteria. They should be done six weeks to six months after complete healing of the CADR (210). It should be performed with the commercialized drug, and whenever possible, also with the pure active products and excipients. If the prescribable form of the drug used by the patient is tested, it should be diluted at 30% in petrolatum or water. The pure drug should be tested, diluted between 1% and 10% wt/wt in pet. or aq., and if needed, tested *as is*. Testing with high concentrations may elicit a relapse of the initial CADR. This is the reason why, when investigating severe CADR, patch tests need to be performed first with very low concentrations, and only if negative responses are observed, should the concentrations applied be increased. Whenever possible, preservatives, coloring agents, and excipients should also be tested *as is* or diluted at 10% in pet., or in the vehicles and concentrations usually proposed for testing in ACD (212). Practically, to include all the ingredients when testing with the prescribable form of the drug, the whole tablet can be crushed and powdered in a mortar, whereas, some of the excipients can be excluded by crushing the tablet after the coating has been scraped off. Similarly, for capsules, the powder can be emptied out and the coating can be wetted and dissolved separately. Similarly, as in ACD testing, the patches are applied to the back for 48 hours and removed one to two hours before the responses are examined. In some situations, such as fixed drug eruptions (FDE) and systemic drug-related intertriginous and flexural exanthem (SDRIFE), it is of value to test the most affected site of the initial CADR (209,213).

The results of the drug patch tests mainly depend on the drug tested and the clinical features of the initial CADR, but there are a few extensive studies that determine their sensitivity and specificity as a complementary tool for drug imputability in CADR. In contrast to the data for SPT, for which there are robust estimates—particularly in the diagnosis of immediate-type hypersensitivity to beta-lactams—(214) in most cases, the figures relating to patch tests are not robust. A working party of the European Society of Contact Dermatitis (ESCD) for the study of skin testing in investigating CADRs, has proposed guidelines for performing these tests, in order to standardize the procedures and also to assess their sensitivity and specificity. In each reported case, the imputability of each drug taken at the onset of the CADR and a highly detailed description and characterization of the dermatitis needs to be given.

The sensitivity and specificity of patch testing in DRESS was assessed in a systematic review, (215) and it was concluded that there was an inconsistency with regard to the positive patch test results between the different causative drugs, within a group of individuals with the same severe reaction pattern. Great variability of results according to the putative responsible drug was also reported: Seventy-two percent positive results for carbamazepine and 0% for allopurinol (209). It is likely that these results are due, at least partially, to drug factors: The logP (octanol : water partition coefficient) for carbamazepine is 1.51 but for allopurinol is only 0.28. Therefore, it can be assumed that allopurinol will have an insufficient skin penetration (209,216). The drugs which have the highest frequencies of positive patch tests are the aromatic anticonvulsants (carbamazepine and phenytoin), the beta-lactams (especially amoxycillin), and amino-penicillins, co-trimoxazole, corticosteroids, diltiazem, diazepam, tetrazepam, and pristinamycin (217–218). In patients with a high imputability of one drug in the onset of their CADR, the drug patch tests had positive results in 43% of 72 patients (219), 50% of 108 patients (220), 43.9% of 66 patients 221, and 31.7% of 197 patients (222). The relatively poor validity of patch testing in the investigation of CADRs, when compared with its use in the study of ACD may be related, but not limited, to several factors, including: (*i*) The capacity for skin penetration of the drug, which is in turn correlated with its lipophilicity and molecular weight, (*ii*) the nature of the true antigenic moiety, which may be a drug metabolite that is not formed in the skin when the patch test with the native drug is applied, (*iii*) the nature of the immune mechanism on the basis of the CADR, which may not be revealed through patch testing, or (*iv*) concomitant factors that may induce a transitory oral drug intolerance—such as viral infections—which are not present at the time of testing.

Therefore, a negative drug skin test does not exclude the responsibility of a drug in the CADR. It is recommended to perform the test with various vehicles, (at least pet. and aq.), to use both the pure and the commercial form of the drug, as well as its excipients,

and to carry out several readings, including immediate (20 minutes) and delayed readings (D2, D4, and D7). Sometimes intradermal testing with delayed reading may be helpful. Finally, when there is a strong suspicion of causality, a systemic challenge needs to be considered. On the other hand, false-positive results have also been observed on drug patch testing, emphasizing the necessity to compare the skin test results with those obtained in negative controls (223).

DIAGNOSTIC VALUE OF PATCH TESTING: RECOMMENDATIONS FOR IMPROVEMENT

Further standardization and refinement in patch testing materials and methodology are unquestionably required. The strategy for maximizing the efficacy and accuracy of patch testing includes the adoption of strict criteria for the selection of patients, further standardization of the patch technique, improved use of dose-response assessments, and most of all, refined and rigorous procedures for the assessment of the clinical relevance of patch test reactions. The stability of many patch test allergens needs to be improved and consistency is also required concerning the allergen dosage. In the TRUE Test system, the major patch test variables are standardized, but unfortunately, only some allergens are available nowadays. It is expected that more allergens can be added to this system. This needs special consideration. To address the problem of false-positive and false-negative patch test reactions, we should produce definite pretest probabilities of fulfilling the case definition for ACD through a comprehensive clinical history and complete physical examination before performing the patch test. Patch test concentrations are selected as the highest concentrations that can be used without inducing an irritant reaction, but it is still capable of evoking an allergic response in sensitized subjects. However, the threshold for irritancy shows a huge variance among individuals. Similarly, delayed hypersensitivity is not an ”all or none” phenomenon, and there are large inter-individual variations in the amount of allergen required to elicit an allergic reaction. Therefore, using a range of allergen concentrations, rather than a single concentration, should be more discriminating. A judicious use of dilution testing will help us to distinguish the false-positive patch test reactions due to irritancy, as well as establishing the sensitization threshold for true allergic reactions. Ascertaining the patient’s degree of sensitization may have important practical implications vis à vis the implementation of rational avoidance measures. Other procedures that may be useful in special situations include testing with different occlusion times, testing with different vehicles, or with different chamber size.

Finally, we should refine and standardize the patch testing technique in the diagnostic study of other allergic disorders, such as, atopic dermatitis, and food, drug, and respiratory allergies.

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48 Diagnostic tests in dermatology: Patch and photopatch testing and contact urticaria

Ludivine J. Bernard, Antti I. Lauerma, and Howard I. Maibach

INTRODUCTION

Diagnostic in vivo skin tests are used in dermatology to detect and define the possible exogenous chemical agent that causes a skin disorder, and hence are critical in their scientific documentation. These chemical agents often cause skin disorders by hypersensitivity mechanisms, which can thus be diagnosed by a provocative test (1). The anatomical advantage of studying skin disorders is that the skin is a foremost frontier of the human body and, therefore, easily accessible for testing. Although differences in the reactivity of different skin sites exist, many causative agents may be tested locally on one skin site, thus exposing only limited areas of skin to the diagnostic procedures. Such procedures include patch, intradermal, prick, scratch, scratch-chamber, open, photo, photopatch, and provocative use tests. In cases of some generalized skin reactions, however, systemic exposure to the external agent may be necessary for diagnosis.

The value of diagnostic tests is identification of the causative agent, which enables restarting of those chemicals or medications not responsible for the eruption. This chapter briefly describes the in vivo test methods used for making diagnoses of skin disorders. The skin disorders in which such tests are useful include drug eruptions, allergic and photoallergic contact dermatitis, and immediate contact reactions (contact urticaria), and possibly sensory (subjective) irritation (Table 48.1).

DRUG ERUPTIONS

Drug eruptions are a heterogeneous class of adverse skin reactions due to ingestion or injection of therapeutic drugs. The drug eruptions should ideally be diagnosed through systemic re-challenge, because many factors (e.g., systemic drug metabolism) may contribute to the process, and skin tests therefore are not as reliable. Because systemic challenge is not always easy to perform, skin tests may, however, precede such challenges, according to reaction type. If skin tests do not provide information about the causative agent and a medication needs to be restarted, the next step is a controlled drug re-challenge, preferably in a hospital environment (2–4)

The choice of provocation protocols depends on the type of reaction involved. Although much work has been directed toward classifying drug eruptions and elucidating their mechanisms, they are still not well understood. Many of them are presumably mediated by immunological mechanisms, but there are also nonimmunological drug eruptions, idiosyncrasies, in genetically predisposed persons. In cases of nonimmunological drug eruptions, skin tests are usually negative, and systemic provocations are also often negative (Tables 48.2 and 48.3). Immunological drug eruptions may be classified into the four reaction types according to Coombs and Gell (5).

Anaphylactic (Coombs–Gell type I) reactions include anaphylaxis, urticaria, and angioneurotic edema. They are usually mediated by immunoglobulin E (IgE) antibodies. Penicillin is one well-known causative agent for type I reactions. Prick (Table 48.4) and scratch (Table 48.5) tests are used in diagnosis of type I reactions and are a relatively safe way of detecting the causative agent. Intradermal tests (Table 48.6) may also be used in such cases, although a much larger amount of the antigen is introduced into the body, which makes systemic reactions more likely. Also, in vitro tests such as the radioallergosorbent test (RAST) are used in diagnosis (5). Because type I reactions are potentially life-threatening, systemic challenges (Tables 48.2 and 48.3) (2), if done, should be performed with extreme care, starting with very low doses, under hospital conditions. A physician should always be readily available, and the patient should be monitored frequently.

Cytotoxic (type II) reactions are mediated by cytotoxic mechanisms: quinine and quinidine are examples of causative agents. Patch tests may be attempted before systemic challenges (Tables 48.2 and 48.3) (2), for example, in the case of thrombocytopenicpurpura caused by carbromal or bromisovalum (5). Pichler has added basic observations to refine these classifications (Table 48.7) (6).

Immune complex-mediated (type II) reactions include Arthus and vasculitic reactions. Type III reactions are mediated by immunoglobulins, complement, and the antigen itself, which form complexes. For example, sulfa preparations, pyrazolones, and hydantoin derivatives have caused vascular purpura via type III mechanisms. Intradermal tests (Table 48.6) may be tried for diagnosis before systemic challenges (Tables 48.2 and 48.3) (5).

Delayed hypersensitivity (type IV) reactions are cell-mediated immune reactions involving the antigen, antigen-presenting cells, and T lymphocytes. Drug reactions of this type are often maculopapular or eczematous, although photoallergic reactions and fixed drug eruptions are also presumably mediated by type IV mechanisms. Other type IV reactions include some cases of erythroderma, exfoliative dermatitis, lichenoid and vesicobullous eruptions, erythema exudativum multiforme, and toxic epidermal necrolysis. Type IV reactions may be detected by patch tests with the causative agent (Table 48.8) (7). In the case of a fixed drug eruption, in which the reaction reoccurs in the same skin site every

TABLE 48.1
Chemically Related Skin Disorders Diagnosable through Diagnostic Testing

Disorder	Mechanism	Test method
Drug eruption	Type I	Prick test or open test Scratch test Intradermal test
		Systemic challenge Patch test
		Systemic challenge Intradermal test
		Systemic challenge Patch test
	Type II Type III	Systemic challenge
	Type IV	Systemic challenge
	Nonimmunological	
Allergic contact dermatitis	Type IV	Patch test Intradermal test
		Open test or provocative use test (repeated open application test)
Contact urticaria syndrome (immediate contact reaction)	Type I	Open test (single application) Prick test Scratch test Scratch-chamber test
	Nonimmunological	Open test (single application)
Subjective irritation	Unknown	Lactic acid test Open test (single application)

[a]Types I–IV: Coombs–Gell classification of immunological mechanisms.

TABLE 48.2
Systemic Challenge: Protocol

The patient should be monitored under hospital conditions and emergency resuscitation equipment should be available throughout the study. Especially if the initial drug eruption was strong, challenge should be started at a low dose, that is, no more than one-tenth of the initial dose.

A dose of the suspected drug is given orally in the morning. The patient's skin, temperature, pulse, and other signs are followed at 1-h intervals for 10 hrs and recorded. If no reactions appear during 24 hrs, the challenge is repeated at a higher dose (e.g., one-third of the initial dose) the next morning.

If no reactions appear on day 1 and 2, then on the third morning a full therapeutic dose is given as a third challenge. If necessary, different drug challenges may be repeated every 24 hrs.

Note: See Ref. 2 for detailed instructions. The publication provides an unequaled clinical experience, and offers many valuable short-cuts in making scientifically based diagnoses.

TABLE 48.3
Systemic Challenge: Precautions

Challenge is not advisable if the patient has had: Anaphylaxis Toxic epidermal necrolysis (TEN) Stevens–Johnson syndrome or erythema multiforme systemic lupus erythematosus-like reaction

Extreme care should be exercised if the patient has had: urticaria asthma any other immediate-type reaction, fixed drug eruption or its most severe form: generalized bullous fixed drug eruption (special variant of TEN)

Usually performed 1–2 months after the original eruption, except in severe reactions, when a longer interval (6 months–1 year) is advisable. Minimum provocative dose is generally less than one single therapeutic dose, except that in cases of severe bullous fixed drug eruption the initial test dose must be smaller (i.e., one-tenth to one-fourth of a single therapeutic dose).

Note: See Ref. 2 for detailed instructions.

TABLE 48.4
Prick Test

Materials: (1) Allergens in vehicles. (2) Vehicle (negative control). (3) Histamine in 0.9% NaCI (positive control). (4) Prick lancets.

Method: One drop of each test allergen, vehicle, and histamine control is applied to the volar aspects of forearms. The test site is pierced with a lancet to introduce the allergen into the skin.

Reading time: 15–30 min

Interpretation: An edematous reaction (wheal) of at least 3 mm in diameter and at least half the size of the histamine control is considered positive, in the absence of such reaction in the vehicle control.

Precautions: General anaphylaxis not very likely, because of the small amount of allergen introduced, but a physician should always be available for such occurrences. The patient should not leave the premises during the first 30 min after the test.

Controls: required.

TABLE 48.5
Scratch Test

Materials: (1) Allergens in vehicles. (2) Vehicle (negative control). (3) Histamine in 0.9% NaCl (positive control). (4) Needles.

Method: One drop of each test allergen, vehicle, and histamine control is applied to the volar aspects of forearms or back, and needles are used to scratch the skin slightly at these sites.

Reading time: up to 30 min

Interpretation: Difficult because of the unstandardized procedure. Edematous reaction at least as wide as the histamine control is considered positive in the absence of such reaction in the vehicle control.

Precautions: as with prick test.

Controls: required.

time the drug is ingested, the patch test should be done in that particular skin site for a positive result (8). For photosensitivity reactions, photo (Table 48.9), or photopatch tests (Table 48.10) should be done (9). A negative patch test does not rule out the possibility that the tested drug may be causative. This is because patch testing involves potential limitations, such as insufficient penetration. In this case a systemic provocation (Table 48.2 and 48.3) should be considered (2).

CONTACT DERMATITIS

Contact dermatitis is commonly divided into irritant contact dermatitis and allergic contact dermatitis. *Irritant contact dermatitis*, the more common of the two, is initiated by nonimmunological toxic mechanisms and is not diagnosed by patch testing, while allergic contact dermatitis is. In *allergic contact dermatitis* the patient becomes topically sensitized to a low molecular weight hapten and in subsequent topical contact develops an eczematous skin reaction, which is mediated by delayed hypersensitivity (type IV) mechanisms. Allergic contact dermatitis is diagnosed with patch tests (Table 48.8), intradermal tests (Table 48.6), or open tests (repeated application) (Table 48.11).

Of these three methods, patch testing is the most common and standardized. The problems involved in patch testing are insufficient penetration of the allergenic compound, which may result in false-negative results and irritation from the test compound, which may cause a false-positive result. Also, patch testing may cause a worsening of eczema in other skin sites (excited skin syndrome) or active sensitization to patch compounds (10).

Two widely used methods for patch testing exist: the Finn chamber and thin-layer rapid use epicutaneous (TRUE) test methods. Both have been shown to be reliable, especially when stronger reactions (contact allergies) are investigated (11). The TRUE test is easier to handle as it is ready to use. However, the Finn chamber method provides more flexibility for the dermatologist and the allergist to test substances not in routine patch test use. Regardless of the test method, the most important factor in successful patch testing is the experience and skill of the interpreter.

A standard patch test series is shown in Table 48.12. It is the standard series of the International Contact Dermatitis Research Group and the European Environmental and Contact Dermatitis Research Group (12). A standard patch-test series has been compiled to represent the most commonly encountered contact allergens, and it is meant to act as a screening tray. Its content is subject to change due to research findings about contact allergy (12). A multitude of other patch-test series is available when the causative agents of the individual patient's contact dermatitis are known better; these include, for example, patch test series for preservatives, rubber chemicals, topical drugs, and clothing chemicals. There are also special series to investigate occupational contact allergies in, for example, dental personnel or hairdressers.

Patch tests should be applied on the back for 48 hours and be read after removal. A second reading 24–48h after patch removal is necessary, as irritant reactions, which are often easily misinterpreted as allergic, often tend to fade during the third and fourth day, while allergic reactions tend to persist. Additionally, with some allergens, such as corticosteroids and neomycin, late reactions often occur, possibly because of low percutaneous penetration. Therefore, a third reading approximately 1 week after patch application may be advisable, although this may be difficult to perform routinely in practice.

Intradermal testing (Table 48.6) is of value in diagnosing hydrocortisone contact allergy (13); see (14) for a review of intradermal testing for allergic contact dermatitis. Open tests or repeated open application tests (Table 48.11) are not as sensitive as patch or intradermal tests, possibly because of insufficient penetration of the compound under unoccluded conditions (15,16).

TABLE 48.6
Intradermal Test

Materials: (1) Allergens in isotonic solution vehicles. (2) Solution vehicle (negative control). (3) Tuberculin (1 cc) syringes and needles.
Method: 0.05–0.1 mL of allergen solution and vehicle solution is applied intradermally to the skin of the volar aspects of forearms.
Reading time: 30 min, 24 hrs, and 48 hrs
Interpretation: Erythematous and edematous reaction at 30 min is suggestive of immediate type (type I) allergy in the absence of such a reaction in the vehicle control.
Arthus reaction with polymorphonuclear leukocyte infiltration appearing in 2–4 hrs, which may progress into necrosis in hours or days, suggests cytotoxic (type III) reaction.
Erythema and edema of at least 5 mm in diameter at 48 hrs indicates delayed-type hypersensitivity (type IV), for example, contact allergy.
Precautions: The risk of general anaphylaxis is higher than in prick or scratch tests because of larger amount of allergen introduced; therefore, a physician should always be available for such occurrences. The risk is greater in asthmatic patients. The patient should not leave the premises during the first 30 min after the test.
Controls: required.

CONTACT URTICARIA SYNDROME: IMMEDIATE CONTACT REACTIONS

Contact urticaria syndrome includes a group of skin reactions, that is, immediate contact reactions, which usually appear within 1 hour of skin contact with the causative agent. Immediate skin reactions are divided into immunological [immunoglobulin E (IgE) mediated] and nonimmunological immediate contact reactions. The symptoms range from mere itching and tingling to local wheal and flare. In cases of intense sensitivity, a generalized urticaria, systemic symptoms, and even anaphylaxis (contact urticaria syndrome) may occur (17).

TABLE 48.7
Relationship of clinical Symptoms to Drug Reactivity

Extended coombs and gell classification	Type of immune response	Pathologic characteristics	Clinical symptom	Covalent and noncovalent drag bindings	Cell type
Type I	IgE	Mast-cell degrnulation	Vrticaria, anaphylaxis	Covalent Drag Bindings	T cells
Type IVa	Th 1 (IFN-γ)	Monocyte activation	Eczema	Noncovalent Drag Bindings	T cells
Type IVb	Th 2 (IL-5 and IL-4)	Eosinophillic inflammation	Macuiopapuiar exanthema, bullous exanthema	Noncovalent Drag Bindings	T cells
Type IVc	CTL (perforin and Granzyme B)	CD4- or CD8-mediated killing of cells (i.e., keratin	Macuiopapuiar exanthema, bullous exanthema, eczema, pustular exanthema	Noncovalent Drag Bindings	T cells
Type IVd	T cells (IL-8)	Neutrophil recruitment and activation	Pustular exanthema	Covalent and noncovalent drug binding	T cells

Modified from Ref. 6

Immunological immediate contact reactions are usually urticarial, although they may range from mere tingling in the skin to a generalized anaphylactic reaction in the whole body. Immunological immediate reactions are Coombs–Gell type I reactions mediated mainly via allergen-specific IgE bound to skin mast cells. Coupling of membrane-bound IgE by allergen causes mast cells to liberate histamine, which with other inflammatory mediators makes skin vessels permeable, and edema (urticaria) results. The sensitization in IgE-mediated contact urticaria may occur through skin or possibly the respiratory or gastrointestinal tract. Exposure through skin is the most likely route in occupational latex allergy in health personnel. The provocative in vivo methods usually performed first are a prick test (Table 48.4),

TABLE 48.8
Patch Test

Materials: (1) Allergens in vehicle (e.g., petrolatum, ethanol, water). (2) Vehicles. (3) Aluminum chambers (Finn chamber), Scanpor tape, and filter papers (for solutions). Or readymade patch test series (TRUE test).
Method: Patches on tape or ready-made patch test series are applied on intact skin of the back. Filter papers are used for solutions: 17 μL of allergen in vehicle is used for each patch. Ready-made patch series is applied as is on similar skin sites. The patches are removed after 48 h.
Reading time: 48 h and 96 h
Interpretation: Erythema and edema or more is positive. Distinguishing between allergic and irritant reaction is important. If the reaction spreads across the boundaries of the patch site, the reaction is more likely to be allergic, if the reaction peaks at 48 h and starts to fade rapidly after that, it may be irritant.
Precautions: Intense skin reactions possible: these can be treated with topical glucocorticosteroids. Active sensitizationpossible.
Controls: required.

TABLE 48.9
Photo Test

Materials: Ultraviolet (UV) radiation source.
Method: Minimal erythema dose (MED) of UVA or UVB is measured (1) while the subject is taking the suspected medication and (2) after discontinuing the same medication.
Interpretation: If MED (UVA or UVB) is much lower while the subject is taking the medication, this suggests a photosensitive (phototoxic or photoallergic) reaction to the drug.

TABLE 48.10
Photopatch Test

Materials: (1) Ultraviolet (UV) radiation source. (2) Patch test materials (Table 48.9).
Method: Two sets of patch test are applied for 48 h. After removal, one set is irradiated with UVA at a dose below minimal erythema dose (MED) (5–10 J/cm2 or 50% of MED, whichever is smaller), and the other set is protected from UV dose.
Reading time: 48 and 96 h
Interpretation: Reaction only at irradiated site suggests photoallergy. Reaction at both sites suggests contact allergy. Reaction at both sites and a much stronger reaction at the irradiated site suggests both contact allergy and photoallergy.
Controls: required.

TABLE 48.11
Provocative Use Test (Open Test or Repeated Open Application Test)

Materials: (1) Allergen in vehicle (petrolatum, ethanol, water) (2) Vehicle (3) Cotton-tipped applicators or other devices to spread the preparations
Method: Patient applies allergen and vehicle on antecubital fossa (outpatient) or shoulder regions of upper back, twice a day, for 14 days or until a positive reaction appears.
Reading time: Patient reports if positive reaction appears. At day 7 and 14, the patient returns for reading of the test site.
Interpretation: Erythema and edema or more is positive.
Precautions: Active sensitization possible, but not yet documented.
Controls: may be required.

TABLE 48.12
Minimal Baseline Series [of the International Contact Dermatitis Research Group (update 2011)]

2-Mercaptobenzothiazole 2%
para-Phenylenediamine (4-phenylenediamine) 1%
4-tert-Butylphenol formaldehyde resin 1%
Budesonide 0.01%
Carba mix 3%MCI/MI (Kathon CG{) 0.02%
Cobalt chloride 1%
Colophony 20%
Compositae mix 5%
Diazolidinylurea 2%
Epoxy resin 1%
Formaldehyde (formalin) 2%
Fragrance mix I 8%
Fragrance mix II 14%
Hydrocortisone-17-butyrate 1%
Hydroxyisohexyl 3-cyclohexene carboxaldehyde (Lyral1) 5%
Imidazolidinylurea (imid urea) 2%
Lanolin alcohol 30%
Mercapto mix 2%
Methyldibromoglutaronitrile 0.3%
Methylisothiazolinone 0.01%
Myroxilon pereirae resin (Balsam of Peru) 25%
N-Isopropyl-N-phenyl-4-phenylenediamine 0.1%
Neomycin sulfate 20%
Nickel sulfate 2.5%
Paraben mix 16%
Potassium dichromate 0.5%
Quaternium-15 2%
Sesquiterpene lactone mix 0.1%
Thiuram mix 1%
Tixocortol-21-pivalate 0.1%
Tosylamide/formaldehyde resin 10%

Source: From Ref. 20

TABLE 48.13
Scratch-Chamber Test

Materials: (1) Scratch test materials (Table 48.5). (2) Chambers.
Method: As with scratch test, but scratch sites are covered with aluminum chambers for 15 min.
Reading time: 30 min.
Interpretation: See Table 48.5.
Precautions: as with prick and scratch tests.
Controls: required.

TABLE 48.14
Open Test (Single Application) for Contact Urticaria Syndrome Immediate Contact Reactions

Materials: (1) Allergen in vehicle (petrolatum, ethanol, water). (2) Vehicle. (3) Cotton-tipped applicators or other devices to spread the preparations.
Method: Allergen and vehicle are applied to skin.
Reading time: Up to 1 h
Interpretation: Urticarial reaction is positive.
Precautions: See Table 48.4.
Controls: Required to aid in disciminating immunological (ICU) from nonimmunological contact urticaria (NICU); in NICU, the reaction will be noted in most controls.

scratch test (Table 48.5), and scratch-chamber test (Table 48.13) (21). However, the test method simulating the clinical contact situation more realistically is the open application test (single application) (Table 48.14). A previously affected skin site is more sensitive to immunological skin reactions than a nonaffected site. In addition to in vivo methods, the diagnosis of immunological immediate-contact reactions can be done with RAST, which detects antigen-specific lgE molecules from the patient's serum (17).

Nonimmunological immediate contact reactions range from erythema to urticaria and occur in persons not sensitized to the compounds (17). Nonimmunological contact reactions are probably more common than immunological contact reactions. They are possibly due to the causative agent's ability to release inflammatory mediators, such as histamine, prostaglandins, and leukotrienes from skin cells without the participation of IgE molecules. Agents capable of causing nonimmunological contact reactions are numerous: the most potent and best-studied agents are benzoic acid, sorbic acid, cinnamic aldehyde, and nicotinic acid esters. The test for diagnosis of NICU is the open application test (single application) (Table 48.14).

SUBJECTIVE IRRITATION

Although sensory (subjective) irritation is not fully characterized, there is evidence for a group of such persons, known as "stingers" (18). The lactic acid test (Table 48.15) has been used experimentally to distinguish between "stingers," who more often have subjective irritation, and "nonstingers" (19).

TABLE 48.15
Lactic Acid Test: Model for Sensory Irritation

Materials:
(1) Facial sauna.
(2) 5% Lactic acid in water.
(3) Vehicle (water).
(4) Soap, paper towels, cotton-tipped applicators.
Method: Facial area below eyes is cleansed with soap, paper towels, and water, rinsed with water, and patted dry. Face is exposed to sauna heat for 15 min. Moisture of face is blotted away. Lactic acid in water is rubbed on one side of face (cheek) and water on other. Face is exposed to sauna again.
Reading time: 2 and 5 min after second sauna exposure.
Interpretation: Any subjective sensation is graded by patient: 0 = none; 1 = slight; 2 = moderate; 3 severe. If cumulative score of two time points is 3 or more, patient is a "stinger."
Precautions: irritation may occur.

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49 Photoirritation (phototoxicity or phototoxic dermatitis)

Dena Elkeeb and Howard I. Maibach

INTRODUCTION

Cutaneous photosensitivity induced by exogenous agents is a well-documented phenomenon. It is defined as an undesirable pharmacological effect that occurs upon simultaneous exposure to photosensitizing chemical or drug and radiation of the appropriate wavelength. Drug-induced phototoxicity is classified as either phototoxic or photoallergic. Multiple chemicals, such as psoralens, fluorescein dye, some thiazide diuretics, and some fluoroquinolones are able to produce both types of cutaneous reactions. It may be difficult to distinguish between these entities, however, they are pathophysiologically distinct processes.

Phototoxicity is much more frequently encountered than photoallergy. It is typically an acute (within minutes to hours), chemically induced nonimmunologic skin irritation requiring light (photoirritation), which is prominent in sun exposed areas, and clinically resembles exaggerated sunburn. Edema, pruritis, erythema, increased skin temperature, vesiculation, and desquamation may be present. These signs may be followed by long-lasting hyperpigmentation. In the classic form, a large amount of chemical or drug exposure is necessary to induce a phototoxic reaction. Histamine, kinins, and arachidonic acid derivatives such as prostaglandins are released during the inflammatory processes. Histologic changes resemble those that would be seen in sunburned skin with epidermal dyskeratosis and vacuolation, as well as dermal edema and vascular changes. Mononuclear infiltrate may be evident.

Photoallergic reactions are much rarer. In contrast with phototoxic reactions, photoallergies usually appear between 24 and 72 hours after exposure to a small amount of the exogenous chemical. Cutaneous manifestations resemble acute, subacute, or chronic dermatitis with significant pruritis, and affected areas may spread beyond areas of sun exposure. Photoallergy requires previous sensitization to the agent and is believed to be immune mediated. Reactions may result from cross-reaction between related chemicals. After drug cessation, re-exposure to sunlight may cause a reoccurence of the reaction. This phenomenon does not occur with phototoxic agents. Histologic changes include epidermal spongiosis, pen- vascular lymphoidosis, and mononuclear exocytosis, which may resemble allergic contact dermatitis.

The degree of photosensitivity among various individuals may vary. Several factors may influence that, such as exposed location, quality of the drug/chemical, pharmaceutical vehicle, ultraviolet (UV) spectrum, thickness of the skin, and degree of melanin pigmentation.

Clinical identification of photosensitivity reactions requires knowledge about skin effects of photosensitizing chemicals and clinical insight gained from practical experience. However, classic morphologic aspects of photosensitivity are not always apparent; prompt and accurate identification of phototoxic and photoallergic dermatoses induced by oral agents may be a challenge to the clinician.

PHOTOSENSITIZING AGENTS

Naturally occurring plant-derived furocoumarins, including psoralen, 5-methoxypsoralen (bergapten), 8-methoxypsoralen (xanthotoxin), angelicin, and others, constitute an important class of phototoxic chemicals. Bergapten, psoralen, and xanthotoxin are among the more commonly encountered photo-toxic agents.

Psoralens are naturally occurring and are synthesized by plants of the Rutaceae (common rue, gas plant, Persian limes, bergamot) and Umbelliferae (fennel, dill, wild carrot, cow parsnip) (1). They also occur in a wide variety of other plants, such as parsley, celery, and citrus fruits (2,1,3). Phototoxicity reactions have been reported to psoralen-containing sweet oranges (4) and to common rue (Rata graviolens) (5) as well as Common lambs-quarters (Chenopodium Album) (6).

Bergapten is the active component of bergamot oil and is a well-known perfume ingredient whose toxic skin effects have been accorded the name berlock dermatitis. Based on results of their studies of perfume phototoxicity (7), suggested that perfume should contain no more than 0.3% bergamot, which is equivalent to about 0.001% bergapten, to avoid phototoxicity. Their work also established that bergapten was the only one of five components isolated from oil of bergamot that was responsible for phototoxic effects of the parent material. Limettin (5,7-dimethoxycoumarin), although more intensely fluorescent than bergapten, did not prove phototoxic to human skin. Bergapten phototoxicity continues to occur in some countries where betgapten-free bergamot is not used (8), in Norway, from contact with *Heracleum lacinutum* (9–11), and in Denmark from skin contact with *Heracieum mantegazzianum*, the giant hogweed (12).

Phototoxicity potential of essential oils used as cosmetic ingredients was reported by (13). Colorants such as Red K 7054-j and chlorophyllin currently used in cosmetics, foods, and feeds were reported to have phototoxic potentials (14).

Xanthotoxin (8-MOP) is effective in treating vitiligo and psoriasis by oral administration or topical application followed by exposure to UVA psoralen plus UVA light (PUVA photo- therapy). The Ammi majus plant, containing xanthotoxin (8- MOP) in crude

form, has been used therapeutically in Egypt since ancient times (15). However, at present PUVA therapy is considered to have carcinogenic potential and warrants caution. Chronic use of this therapeutic regimen enhances prospects of inducing squamous-cell skin cancer, especially in young patients and in those who are genetically predisposed (16). This potential has resulted in a reduced use of PUVA phototherapy in the United States (17).

There are a number of agents outside of the furocoumarin family that are phototoxic. Coal-tar derivatives produce occupational contact photodermatitis and phototoxicity in industrial workers and road workers. Anthraquinone-based disperse blue 35 dye caused such effects in dye process workers. Radiation in the visible spectrum activates the dye (18). Pyrene, anthracene, and fluoranthrene are strongly phototoxic to guinea pigs (19).

Phenothiazines, such as chlorpromazine, cause photo-toxic effects, which have also been seen with oral therapeutic use of amiodarone, a cardiac antiarrhythmic drug (20). Incidence, time course, and recovery from phototoxic effects of amiodarone in humans were studied by (21). Antimalarials quinine and quinidine appear to be phototoxic, and some of these have been studied in vitro and in vivo (22–24). Cadmium sulfide, used in tattoos for its yellow color, may be phototoxic (25). Thiazide diuretics were shown to have a phototoxic potential in one study (26), but thiazide-induced phototoxicity is actually rare in clinical practice. There have been recent reports of phototoxicity induced by perforatum hypericum, contained in herbal antidepressant St. John's wort (27). This agent may function through mechanisms including inhibition of proteasome function (28).

Tetracyclines, particularly clemethylchlortetracycline, and also doxycycline, chlortetracycline, and tetracycline, are phototoxic when orally ingested (29–31). Doxycycline was reported more potent than dernethylchlortetractracycline or limecycline in one human study (32).

Some fibric acid derivatives, such as fenofibrate, have been reported to exhibit photosensitizing effects in vivo. Bezafibrate and gemfibrozil are mildly phototoxic, and clofibrate has shown not to be phototoxic at all (33). Diltiazem has also shown to cause photoxicity in some case reports (34).

Fluoroquinolone antibiotics have recently proven to be phototoxic (35). There have been a number of controlled trials supporting this phenomenon. Fluoroquinolones differ significantly in their extent of phototoxicity. Recently, in a randomized, placebo-controlled study comparing phototoxicity (36), found sitafloxacin to be mildly phototoxic; enoxacin and sparfloxacin proved to be much more photoactive in white subjects. Levofloxacin and placebo failed to show a phototoxic effect. In contrast, among Asian subjects, sitafloxacin failed to demonstrate significant phototoxicity. A randomized-controlled trial supported the fact that lemofloxacin, but not moxifloxacin had phototoxic effects (37).

It is generally accepted that clinafloxacin » lomefloxacin, sparfloxacin, trovafloxacin, nalidixic acid, ofloxacin, ciprofloxacin > enoxacin, norfloxacin (38). Perfloxacin and sparfloxacin also appear to result in higher amounts of phototoxicity than ciprofloxacin (39). Ciprofloxacin was found to induce phototoxic effects at ambient levels of sunlight (40). It is generally believed that levofloxacin and moxifloxacin are among the least phototoxic drugs in this class (41).

Antifungals such as voriconazole, a broad spectrum triazole were suggested by multiple case reports to have phototoxic properties (42–44). Griseofulvin was found to have less severe phototoxic potential with its single oral formulation in contrast with its dermal administration (45).

Antimicrobials, such as sulfonamides, and some fluoroquinolones (enoxacin and lomefloxacin) cause a cutaneous photoallergic reaction, as can sunscreen ingredients, most notably para-aminobenzoic acid (PABA) and its derivatives, and fragrances such as musk ambrette. As previously mentioned, thazides, fluorescein dye, and psoralens are photo- toxic, as well as photoallergic.

Multiple case reports suggest that pyridoxine hydrochloride (vitamin 136), may have some photoallergenic activity and have been photopatch tested as positive for this agent (46,47).

Several psychiatric medications including tricyclics, carbamazepine, and benzodiazepines have shown to be cutaneous photoallergens.

Other miscellaneous drugs implicated as photoallergens include amantidine, dapsone, nifedipine, and isotretinoin. However, for a number of these agents, formal data proving their photoallergenic potential are lacking.

NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

Nonsteroidal anti-inflammatory drugs (NSAIDs) were the subject of extensive investigations for phototoxic potential following reports that benoxaprofen, a suspended British anti- rheumatic NSAID, has this capacity (48–51). In vitro studies with sheep erythrocytes or human leukocytes suggested a photo-toxic potential (51,52). NSAIDs that are structurally related to propionic acid have been shown to possess phototoxic potential, whereas certain other types of NSAIDs, such as tenoxicam and pi roxicam were not experimentally phototoxic by in vivo or in vitro test methods (51,53,54). The propionic acid-derived NSAIDs produce unique immediate wheal and are, in contrast, with a much delayed exaggerated sunburn response that typifies psoralen phototoxicity.

Although piroxicam is not phototoxic under experimental conditions involving human test conditions (53), it has been implicated as a possible clinical photoallergic or phototoxic photosensitizer. One explanation for the unexpected photoactivity of piroxicam in skin is that a metabolite of piroxicam is indeed phototoxic when isolated and tested on human mononuclear cells in vitro (54). These positive findings and likely explanation are related to the production of singlet oxygen, as indicated by emission at 1270 nm when the suspect metabolite was irradiated with UV in vitro (54,55).

Other propionic acid–derived NSAIDs associated with an immediate phototoxic response are nabumetone, naproxen, and tiaprofenic acid (53,56).

Carprofen (57), ketoprofen (58,59), benzydamine hydrochloride, topical tiaprofenic acid, suprofen, and possibly piroxicam appear to be photoallergenic. However, further work may be needed to separate, clarify, and identify three possible outcomes—allergy, photoallergy, and phototoxicity—in studies involving NSAIDs.

The general area of cutaneous reactions to NSAIDs has been extensively reviewed in (60).

MECHANISMS OF PHOTOTOXICITY

Phototoxic and photoallergic chemicals typically exhibit biologic response with the UV area of the electromagnetic spectrum, which is subdivided with arbitrary limits into UVA (320–400 nm), UVB (290–320 nm), and UVC (200–290 nm). UVA represents the less energetic portion of the spectrum and UVC the more energetic (cytotoxic) area. UVA in the range 320–340 nm (UVA2) is more energetic and more skin damaging than UVA in the range 340–400 nm (UVA 1). In vivo, both phototoxicity and photosensitivity are primarily due to UVA range light. However, in vitro, phototoxic agents absorb and are activated by both UVA and UVB wavelengths. The cause of this discrepancy is unknown. Some phototoxic chemicals, such as porphyrins and fluorescein dye, absorb visible light (400–800 nm).

Exogenous phototoxic reactions are initiated when a photoactive chemical (one capable of absorbing UV radiation) or one of its metabolites enter viable skin cells. The photoactive chemical may reach the skin via the topical route or it may reach the skin systemically following ingestion or parenteral administration. Some systemically administered and possibly topical chemicals may require metabolic conversion to become photoactive, such as Pitavastatin and Fluvastatin (61,62). Certain drugs have their metabolite causing more photosensitivity reaction than the parent drug itself (63,64). When the photoactive chemical is in the skin, appropriate wavelengths of light penetrate the skin and subsequently photons are absorbed, and thereby excite electrons in the phototoxic chemical. This process may lead to the formation of unstable singlet or triplet states. As these molecules transfer energy to achieve a more stable state, the transferred energy induces cellular damage and generates inflammatory mediators.

The questions of site and mechanism of action of phototoxic chemicals and the importance of oxygen have been much studied. Some phototoxic agents are oxygen dependant, or photodynamic, whereas others are not. Photodynamic chemicals may transfer their energy to oxygen, exciting it to the singlet or doublet state, thereby exerting phototoxic effects. In its excited state, the photodynamic chemical may react with oxygen and form free radicals. Though mechanisms causing reactions of photoactive drugs are mainly free radical in nature, reactive oxygen species are also involved. Photochemical activity of drugs, such as hydrochlorthiazide, furosemide, chlorpromazine, and some NSAIDs is caused by free radical formation. In other systems, the reactive excited singlet form of oxygen is directly toxic toward lipids and proteins (65).

Studies by (66) have shown that acridine requires oxygen to produce a lethal (phototoxic) effect on mast cells. (Dermal mast cells are known to participate in cutaneous phototoxic responses initiated by UV and visible radiation.) Chlorpromazine is also thought to be activated by a photodynamic process involving molecular oxygen.

Reactive oxygen intermediates may be a main cause of photosensitivity reactions, which can be stopped by agents that block the production of these intermediate products. Antioxidant supplementation may be beneficial in suppressing phototoxic reactions. Vasoactive amines such antihistamine and serotonin may also play a role in cutaneous phototoxic reactions. Eucosanoids, such as prostaglandins and leukotreines, have also been implicated in the process.

(67) showed that toluidine blue requires oxygen to produce its lethal (phototoxic) effect on Sarcina lutea; however, oxygen is not needed for the phototoxic effect of 8-MOP on S. lutea. In addition, it was found that 8-MOP phototoxicity results in damage to cellular DNA, whereas toluidine kills by action on the cell membrane. Psoralens also do not require molecular oxygen to produce phototoxic effects.

Some photoactive chemicals act on cellular DNA (psoralens, may be tricyclics), whereas others act on cellular membranes (tricyclics). Fluoroquinolones may induce DNA breaks and lead to cell death. Keratinocytes may be the most sensitive cells and melanocytes most resistant (68). The differences in phototoxicity potential may be based on differences in substituent placement on the various chemicals (69).

Photoallergic reactions are believed to be cell mediated, with radiation-dependant antigen production, therefore stimulating the immune response. UV energy may cause the drug hapten to find a native protein on epidermal cells, thereby forming a complete photoantigen. When the antigen is formed, formed, the photoallergic process is similar to allergic contact dermatitis, with sensitization of the immune system, and a subsequent cutaneous eruption.

A more complete discussion of mechanisms of photosensitized reactions is given in (70).

ELEMENTS OF THE TEST FOR PHOTOTOXICITY

Tests for phototoxic potential of topically applied chemicals are usually conducted with radiation within the UVA range. Some phototoxic chemicals are activated by wavelengths in the visible spectrum (bikini dermatitis) (71), some by UVB (72,73), and some (doxycycline) are augmented by UV B (74).

Both in vivo and in vitro assays have been developed to evaluate the phototoxic potential of chemicals.

Accurate measurements of radiation intensity and frequency are important prerequisites for work in phototoxicity.

In vivo phototesting procedures include photopatch testing and determination of minimal erythema dose (MED) for UVA and UVB. Photopatch testing may be more useful in detecting photoallergy, and MED may be more useful for testing phototoxic agents. Owing to the difficulties in distinguishing photoallergic from phototoxic reactions with the photopatch test, it is recommended that in practice both types of testing should be performed to ensure comprehensive evaluation.

Among animal models for which photopatch testing has proven useful in predicting human phototoxicity are the mouse, rabbit, swine, guinea pig, squirrel monkey, and hamster, in that approximate order of effectiveness (7,75).

The test material is applied to the skin of a human subject or an animal model (clipped skin of mouse, guinea pig, rabbit, or swine). After a suitable waiting period for skin absorption to take place (several minutes, depending on the rate of skin penetration), the chemical test site is irradiated with UV of appropriate wavelengths. The test site is then examined at 1, 24, 48, and 72 h for evidence of phototoxicity, such as erythema, vesiculation, bullae, and finally hyperpigmentation. A comparison is made between the skin of the test site and control sites (one without chemical and one without light).

Results are modified by factors that affect skin penetration, such as test concentration and vehicle, as well as by duration of exposure and by distance from the irradiation source to the test area.

Some photoirritants (e.g., bergapten) produce clinical phototoxicity when the photoirritant site is irradiated within minutes to 1 h after skin application; with others, irradiation is effective when administered at 24 h.

Phototoxic effects are expected when UV is directed at and absorbed by a phototoxic chemical residing in the skin. This results in a skin reaction with cellular components such as DNA.

One of the earliest indicators of phototoxic potential was based on a paralyzing effect on the cilia of *Paramecium* from acridine plus light, reported by Oscar Raab at the close of the nineteenth century. This test method was later followed by a simpler test involving a lytic effect on red blood cells, as an endpoint for phototoxicity.

Legislations by the regulatory bodies in Europe and the United States aiming to reduce or eliminate animal use for phototoxicity testing has led to the development of alternative in vitro methods of testing such as the 3T3 NRU PT that was validated and adopted by the Organization for Economic Cooperation and Development (OECD) in 2004 for testing of chemicals.

The subject of in vitro assay for phototoxic effects has recently been reviewed in (76).

Recently, OECD prevalidated reconstructed human epidermis models, such as EpiSkin, SkinEthic, and EpiDerm, have demonstrated ability to serve as an adjunct in vitro model to 3T3 NRU for phototoxicity testing (77,78).

Certain models have proven effective in discriminating between phototoxic and nonphototoxic compounds, as compared to in vivo data (13).

Results of in vitro assays may not be directly related to human reaction in vivo as the bioavailability may differ (79,80). Several protocols for use are currently available. In the future, data obtained from these models will likely contribute a wealth of information, thereby increasing our knowledge and understanding of photosensitivity.

HIGHLIGHTS

Important prerequisites for phototoxicity testing:

- Testing materials that absorbs light energy within the same light range.
- Intensity of light (Irradiance): Well-calibrated equipment for measuring radiation, including recognition that with time and use, equipment changes and requires proper upkeep to ensure its quality in performance.
- Light source and filters: Irradiation spectrum should approximate the solar spectrum with appropriate filters to remove the UVC and attenuate the UVB part of the emission spectrum to the levels of ambient sunlight. Window glass is useful in eliminating wavelengths below 320 nm. Natural sunlight is filtered by atmospheric oxygen, ozone, clouds, particulates, and other environmental factors including altitude, so that wavelengths below 290 nm are effectively shielded from reaching the earth's surface. Consequently, radiation sources that deliver highly energetic shorter wavelengths in the UVC range are unlikely to be useful in experimental photosensitivity studies involving humans.

Knowledge about safety in the use of radiation equipment is equally important.

The radiation ranges that are of greatest biologic focus in photosensitivity studies are UVA (320–400 nm), UVB (280–320 nm), and UVC (<280 nm). As the Commision de l'Eclairage recommends 315 nm as the cutoff for UVB rather than 320 nm, it is important that the investigative photobiologist identify the system of use. However, a rationale for using 320 nm rather than 315 nm as the cutoff for UVA is given in (81).

TABLE 49.1
Chemicals, Plants, and Drugs with Phototoxic Potential

Topical dyes—anthraquinone, fluorescein dye, disperse blue 35, eosin, methylene blue, rose Bengal, toluidine blue, cadmium sulfide in tattoos
Cosmetic and food colorants
Essential oils in cosmetics
Fragrances—oil of bergamot
Furocournalins—angeliciii, bergapten, psoralen, 8-methoxypsoralen. 4,5, 8-trimetbylpsoralen
Plant products—celery, figs, limes, hogweed, parsnips, fennel, dill Coal-tar components—acridine, anthracene, benzopyrene, creosote, phenanthrene, pitch, pyridine
Systemic antibiotics—griseofulvin, ketoconazole, voriconazole, nalidixic acid, sulfonamides, ceftazidirne, tetracyclines, fluoroquinolones Chemotherapeutics—dacarbazine, 5-fluorouracil, vinblastine, methotrexate
Drugs—amiodarone, Simvastatin, chlorpromazine, quinine, quinidine, tolbutamide, diltiazem, fibric acid derivatives, hyerpicum perforatum (St. John's wort) Diuretics—hydrochlorothizide, bendroflumethiazide, furosemide Nonsteroidal anti-inflammatories—benoxaprofen, naproxen, piroxicam, tiaprofenic acid, nabumetone
Porphyrins—hematoporphyrin

The first rule of photochemistry is that cells are injured or killed when photons of radiant energy are absorbed and energy is transferred to target molecules (70). Phototoxic effects are therefore produced when absorption wavelengths of the sensitizer are the same as those of the radiant energy source (Grotthus–Draper law).

DNA, RNA, deoxy- or ribodeoxynucleotides, enzymes containing such cofactors, and aromatic and cysteine residues of proteins are typical targets of UV phototoxic damage.

Oxygen may or may not participate in the production of a phototoxic event; however, when oxygen is indeed involved, it is often referred to as a photodynamic action.

Psoralens are among the most frequently encountered phototoxic chemicals, as they are present in many plants. Petroleum products, coal tar, cadmium sulfide, acridines, porphyrins, and other chemicals may also be implicated as causative agents for phototoxic effects. Table 49.1 provides a list of phototoxic chemicals.

Finally, it is suggested that investigators be complete in identifying equipment and methodology that they employ to reduce some of the confusion that may enter and has already entered the literature on this subject as well as standardizing their approach and perform more randomized blinded interlaboratory assays.

CONCLUSIONS

Years of investigative efforts, along with improved methods of measuring and administering radiation, have brought considerable progress in our understanding of various aspects of photosensitivity. We appear to have identified and continue to identify major chemical structures that are currently involved in producing phototoxic and photoallergic effects in humans. We have also gained some insight into some of the mechanisms that are involved. Nevertheless, it is always important to be flexible and aware that time may change some of our present and apparently well-conceived perceptions, as it often does.

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50 Significance of methyl mercury hair analysis: Mercury biomonitoring in human scalp/nude mouse model

Grazyna Zareba and Thomas W. Clarkson

INTRODUCTION

Methyl mercury (MeHg) is a well-known fish contaminant and potential developmental neurotoxin, for which exposure standards are still under debate (1–3). Several ongoing projects aim to determine the effects of low-level MeHg exposure from fish diet during pregnancy and their impact on the unborn child's neurodevelopment (4). In most studies maternal hair has been used as an indicator of mercury fetal exposure (1,4). However, there is ongoing controversy whether hair or whole blood serve as a better indicator of MeHg exposure. The choice of the indicator medium for epidemiologic and clinical studies depends on several factors, such as the concentration measured, the access to the medium, and its ability to predict levels in the target tissue. Human hair as a biomonitoring medium and has several well-established advantages: noninvasive sampling, easy collection, sample stability during storage, accessibility, and most importantly long-term recapitulation of the history of past exposure (5). Nondestructive physical methods, such as X-ray fluorescent spectrometry, are now available that can measure mercury in single strands of hair, and the difference in mercury levels between each strand has been shown to be less than the analytic variance (6). Although some data have indicated that certain hair treatments might remove mercury or possibly add mercury as a contaminant (7,8), a large population study found no evidence of any effect of hair treatment on hair mercury levels (9).

The scalp hair accumulates MeHg to a remarkable degree showing on average, the hair to whole blood concentration ratio as 250:1 (1,10,11). Once incorporated into the hair strand, the concentration of MeHg remains stable. Thus, based on the assumption that hair grows approximately 1 cm per month, hair allows the recapitulation of past exposure for many months from a single sample of hair, depending on the length of the hair sample (12). The ability of hair to concentrate MeHg and to maintain stable concentrations is an important factor when evaluating hair as a biologic index of exposure.

The discovery of the nude mouse in the 1960s as a spontaneously occurring new mutant (13) brought a new model to study many aspects of investigative dermatology. It has been demonstrated that in mice, similar to rats and humans, loss of function of Foxn1, a member of the winged helix/forkhead family of transcription factors, leads to macroscopic nudity and an inborn dysgenesis of the thymus (14,15). Nude [Foxn1(nu)/Foxn1(nu)] mice develop largely normal hair follicles and produce hair shafts. However, presumably because of a lack of certain hair keratins (13), hair shafts fail to penetrate the epidermis and macroscopic nudity generates the misleading impression that nude mice are hairless (15). A comprehensive review on the role of Foxn1 in mammalian skin biology, its expression patterns in the hair follicle, and its influence on hair follicle function have been published by Mecklenburg et al. (15). Today, nude [Foxn1(nu)/Foxn1(nu)] mice have been widely used in immunologic, dermatologic, cosmetic, oncologic, and transplantation research, particularly because of their defect in allo- or xenotransplant rejection. For example, the Foxn1(nu) mice have been used in studies of a regulatory model of keratinocyte differentiation (16).

The Foxn1(nu) mouse model has also proved to be very helpful in many areas of dermatotoxicology. Human skin grafted onto the athymic nude mouse has been validated and used to study in vivo percutaneous absorption and metabolism of several substances in the skin, such as caffeine, benzoic acid, diethyl toluamide, steroids, insecticides, and silicones (17–19). The human scalp/nude mouse model has been used when evaluating iodine biomonitoring (20). Several studies demonstrated that this unique system maintains unchanged morphology and biochemistry of human skin for a long period of time (20–23). Therefore, in the present study the human scalp skin/nude mouse model was characterized and evaluated for the biologic monitoring of MeHg.

METHODOLOGY FOR METHYL MERCURY INCORPORATION INTO HUMAN SCALP/NUDE MOUSE MODEL

Athymic BALB/c nu/nu nude mice (NIH Taconic Farm, Germantown, New York, USA) were housed in facilities with programs accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Human fetal scalp grafts obtained from aborted fetuses, with gestational ages ranging from 18 to 22 weeks (estimated from the heel–toe length standards), were used for transplantation. The skin (8–20 mm in diameter), after dissection from the underlying soft tissue, was placed into RPMI medium containing fungazone (2.5 μg/mL, Squibb, Princeton, New Jersey, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) for 24 hours before being transplanted to the nude mice. Grafts were transplanted subcutaneously using pentobarbital (60 mg/kg) for anesthesia (24).

Multiple biopsies of control human fetal skin, transplanted grafts, and mouse skin were taken every month, fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. To confirm human characteristics of the transplanted scalp throughout the entire study, additional frozen or paraffin sections stained with immunofluorescence and immunoperoxidase techniques with monoclonal antibodies to human class I antigen W 6/32, mouse class I antigen SF 1-1.1.1, and human epidermal keratins KAB3 were used.

Trichograms, morphometric analysis of rate of hair growth, hair density, state of the hair cycle, and hair thickness of human scalp, and hair growing in human grafts ($n = 30$) were performed within 1–6 months after scalp transplantation using a standardized technique under the light microscope (25,26).

After human hair growth was observed in transplanted scalp grafts, mice were administered CH_3HgCl continuously for 2–4 months, using subcutaneously implanted ALZET osmotic pumps (model 2002) at the concentrations 180 μg Hg/ml and 3 mg Hg/ml (72 μg Hg/kg/day and 1.2 mg Hg/kg/day, respectively). Blood and hair samples were collected after 1, 2, and 4 months. The mercury concentration in human hairs grown on the nude mouse was determined using X-ray fluorescence spectrometry by segmental (2 mm) analysis of single strands (6,27). Tissue concentrations were measured by cold vapor atomic absorption analysis (28).

In autoradiographic studies, mice with transplanted human scalp hair were injected intraperitoneally with a single dose of $CH_3\ ^{203}HgCl$ (6 mgHg/kg, the radioactivity 50 μCi/kg body weight). After two days and after four weeks the animals were anesthetized, grafts and mouse skin biopsies were taken and embedded in plastic. Cross and sagittal sections were cut at 2 μm and processed for autoradiography with Kodak NTB-2 film emulsion, exposed for 21 days at 4°C and developed. Sections were poststained with toluidine blue and mounted with Permount. More detailed information on all methods was described by Zareba et al. (23).

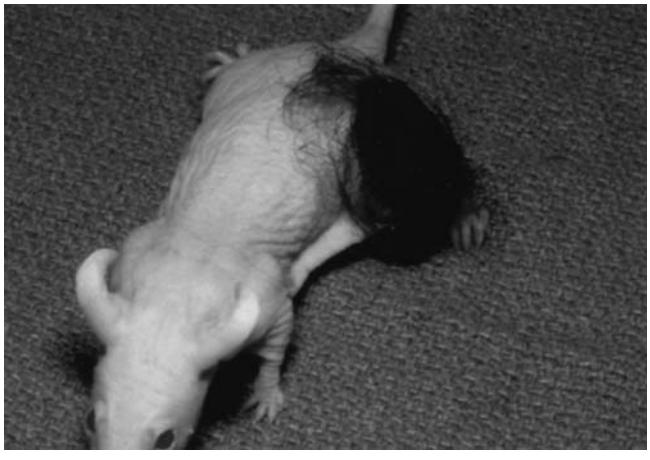

FIGURE 50.1 Human scalp graft six months after transplantation into nude mouse with extensive hair growth (23).

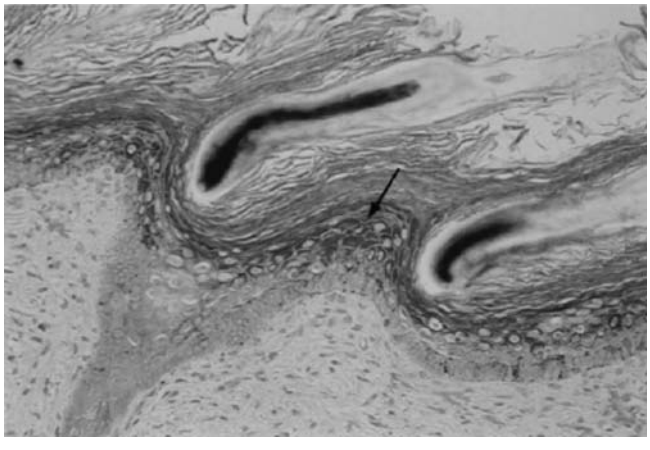

FIGURE 50.2 Histologic section of human skin graft two months after transplantation. The pattern of staining with the KAB3 antibody (arrow indicates suprabasal layer) identifies the human graft and shows that the differentiated state of the epidermis is maintained (23).

CHARACTERISTICS OF HUMAN SCALP/NUDE MOUSE MODEL

Human scalp hair demonstrated extensive hair growth for several months after transplantation (Fig. 50.1). Histologic sections of control fetal scalp skin with an estimated gestational age of 18–19 weeks two months after transplantation showed the full development of the epidermis, dermis, hair follicles, and appendages. The KAB3 antibody staining of the suprabasal layer of the epidermis demonstrated the differentiated state of the epidermis (Fig. 50.2). The preservation of human characteristics of the grafted tissue was verified by additional staining with monoclonal antibodies against the species-specific Class I antigens W 6/32 and SF 1-1.1.1.

The mouse model has been used in several studies on MeHg toxicology due to the similarity of some aspects of mice and human MeHg kinetics, such as brain-to-blood ratios (29–31). However, the mouse is not a satisfactory model to study MeHg incorporation into hair due to the following reasons: (*i*) the hair cycle in a mouse is short (only about three weeks), and does not provide enough time to obtain mercury steady-state levels (in contrast, human scalp hair has a cycle time of several years); and (*ii*) mice demonstrate very different hair growth patterns (wave-type growth vs. mosaic growth in humans). Therefore, the human hair/nude mice model was the best choice to recapitulate the level of MeHg exposure.

TRICHOGRAM OF HUMAN HAIR GROWN ON NUDE MICE

A trichogram of transplanted hair within six months demonstrated that human hair growing on the nude mouse represented values typical for human hair (Table 50.1), with hair growth, density, thickness, and anagen:telogen ratio characteristic for human neonatal scalp hair. It is interesting that morphometric analysis of transplanted hair showed a growth rate of 0.57 cm/mo, close to the hair growth rate of 0.56 cm/mo, observed in infants exposed to phenyl mercury acetate from contaminated diapers (Cernichiari et al., personal communication). Because the human scalp used in these studies can be considered as neonatal hair corresponding to a gestational age of 18–22 weeks during transplantation (32), these data together with previous observations suggest that child human hair shows a slower hair growth than adult hair.

TABLE 50.1
Trichogram (Mean ± SD) of Human Hairs Grown on Nude Mice (23)

Parameter	Mean value	Normal values for human hair[a]
Growth rate (cm/mo)	0.56 ± 0.18	0.8–1.04 0.57[b]
Density (number/cm)	376.2 ± 137.6	300–400
Thickness (%)		
Thin (<0.025 mm)	0	
Medium (0.026–0.050 mm)	88(70–95)	55–85
Thick (>0.050 mm)	12 (10–2)	
State of cycle (%)		
Anagen	82 (68–92)	60–70
Telogen	18 (10–32)	13–15
Anagen:telogen ratio	4.6	5

[a]From Ref. 26.
[b]Cernichiari (personal communication).

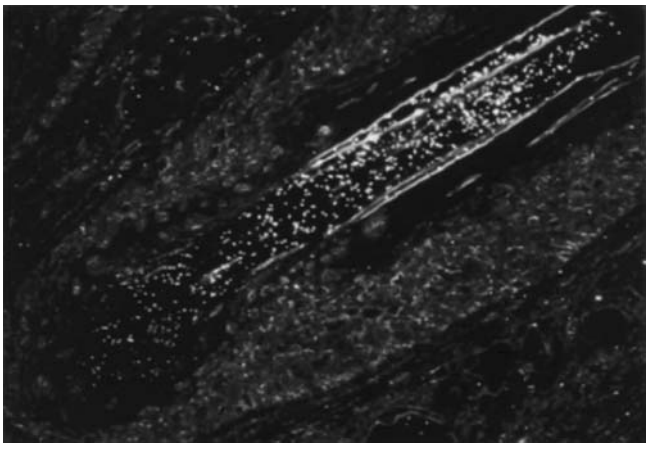

FIGURE 50.3 Autoradiograph of transplanted human scalp one month after methyl mercury administration (6 mgHg/kg i.p.) (longitudinal section, dark field). Mercury uptake predominantly into hair shaft (white silver grains) (23).

METHYL MERCURY INCORPORATION INTO TRANSPLANTED HUMAN HAIR

Mercury uptake predominantly into the human hair follicle as evidenced by the presence of silver grains was demonstrated by autoradiographic studies of human skin transplanted onto nude mice one month after MeHg administration (Fig. 50.3). Most of the mercury was concentrated in the hair shafts, especially in the matrix and the keratogenous zone of the hair follicle. A cross section of the hair follicle evidently demonstrated the same distribution pattern. In the hair shafts, MeHg concentrated along the cuticle and the cortex.

MERCURY CONCENTRATIONS IN HUMAN HAIR AND ANIMAL BLOOD

Mercury hair concentrations after scanning over the hair length with data taken every 2 mm, were two orders of magnitude higher than in blood, and reached a mean hair:blood ratio of 219. An example of MeHg concentrations in grafted human hair and blood of nude mice after four months of continuous exposure (osmotic pumps, 3 mg Hg/ml or 1.2 mg Hg/kg/day) is shown in Figure 50.3.

It has been well documented that in humans the concentration of mercury in newly formed hair is directly proportional to the concentration in blood, with the hair-to-blood ratio of approximately 250:1 (1,2,10,33). In our model we did not expect to obtain exactly the same human hair to mouse blood ratio as for humans because the binding characteristics for MeHg in mouse blood may not be the same as for human blood. Nevertheless, despite this potential difference, at the steady-state levels of mercury in the body (Fig. 50.4), a constant hair:blood ratio of 219:1 (range, 194.5–237.8) was observed at two different exposure levels, which is typical only for human exposure (23). Most animal studies show much lower hair-to-blood ratios (1), probably due to different hair cycles. Even in the Macaque monkey, whose hair growth shows many similarities to human scalp growth, Mottet et al. (34) observed a hair:blood ratio of 124:1, significantly lower than in humans.

When studying mercury incorporation into hair, a question arises if hair may also be an important route of elimination of MeHg from the body. Magos and Clarkson (35) calculated the rate of elimination of MeHg in hair based on existing data on hair physiology (hair growth and density) and on the MeHg deposition parameters in the body (i.e., hair to blood concentration ratio). A quantitative estimate of the contribution of hair to total excretion from the body indicated that hair accounted for only a small fraction, less than 10%, of the total elimination of MeHg from the body.

Another question that arises when studying mercury accumulation in the hair is whether this accumulation exists in the case of different organic species of mercury. Ethyl mercury in thimerosal-containing vaccines, still used in many countries, is one of the most controversial sources of early postnatal exposure to organic mercury. It has been shown that ethyl mercury incorporates into growing animal hair in a similar manner to MeHg (36). When the hair content of methyl and ethyl mercury was plotted against the corresponding blood levels, the uptake into hair of newborn mice (with hair in the growing phase) was identical. This observation has been confirmed by Dórea et al. (37) who found ethyl mercury in the hair of infants exposed to thimerosal-containing vaccines (Hepatitis B and DTaP). However, contrary to organic mercury species, the uptake of inorganic mercury into hair occurs at significantly lower levels (11). In several individuals who were exposed to high doses of inorganic mercury (due to accidental or suicidal exposure), hair levels were elevated, but only one to two orders of magnitude lower than those that would be expected from the same blood levels of MeHg.

MECHANISMS OF MERCURY UPTAKE INTO HUMAN HAIR

Although human scalp hair has been used for over 40 years in mercury research, the mechanisms of MeHg transport and incorporation into hair have not been studied extensively. Theoretically, MeHg can enter the hair follicle through at least three routes: the blood during hair formation, sweat or sebum, and through external contamination. MeHg animal and in vitro studies demonstrated that MeHg enters the epithelial cells of the blood–brain barrier as a complex with the amino acid L-cysteine,

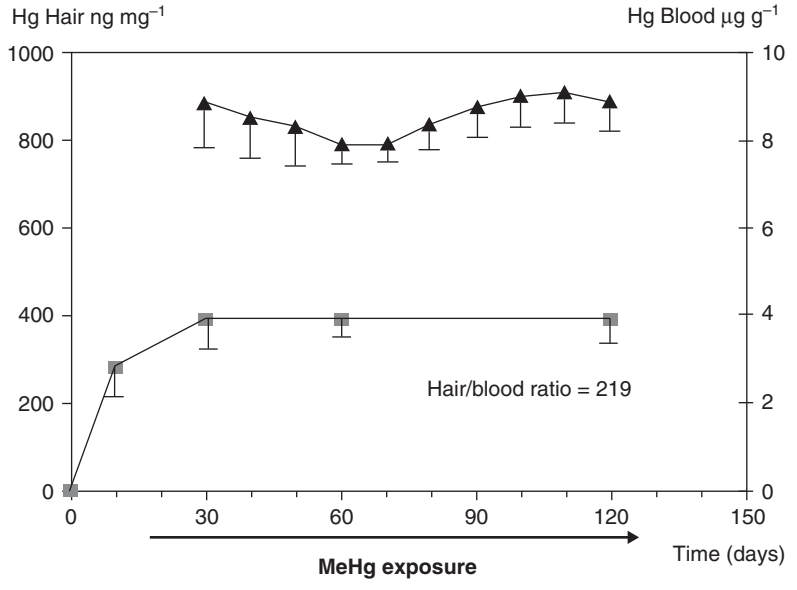

FIGURE 50.4 Mercury concentrations in grafted human hair (mean ± SD) and in blood (mean ± SD) of nude mice after methyl mercury administration (3 mgHg/mL, osmotic pumps) (23).

whose structure is very similar to that of the large neutral amino acid methionine (38,39). Methionine is carried into the cell on the large neutral amino acid carriers. It also may be the main pathway for MeHg incorporation into hair because the large neutral amino acid carriers are present in all mammalian cells actively performing protein synthesis, such as the hair follicle. Based on existing data, it has been speculated that MeHg is transported to the hair follicle through the blood stream and enters the keratinocytes as a MeHg–cysteine complex via the large neutral amino acid carriers (23). Keratinocyte proliferation in hair follicles occurs adjacent to the blood supply (40,41). MeHg can then be incorporated into the hair matrix during the process of keratin synthesis in keratinocytes because of its high affinity to abundant sulfhydryl groups (SH) in the hair protein. Our autoradiographic data (Fig. 50.3) support this mechanism of MeHg uptake into hair in which MeHg incorporation was shown mostly in the keratogenous zone high in SH groups. In previous studies, it has been demonstrated that MeHg as well as ethyl mercury incorporate into newly born mouse hair only during the growing, anagen phase (36,42). Additional confirmation of this hypothesis may come from the studies of Hislop et al. (43) on volunteers taking repeated oral doses of MeHg, showing that the concentrations in 8 mm segments of hair next to the scalp lagged behind the blood concentration by about 20 days. This delay can be explained by the known growth rate of hair assuming MeHg was initially transported from blood into the hair follicle. More detailed information on the mechanism of mercury incorporation into hair has been described in the reviews of Cernichiari et al. (11) and Clarkson and Magos (4).

IMPLICATIONS FOR METHYL MERCURY EPIDEMIOLOGIC STUDIES

Hair has been used as the tissue of choice for biologic monitoring of MeHg exposure in most epidemiologic studies. However, there is no universal agreement as to which medium, that is, hair or blood, is a better index of MeHg exposure.

Several studies have now been published on the potential adverse effects of prenatal exposure to MeHg on child development (for a review, see Clarkson and Magos, (4)). Although measures of child development follow well-established methods, the measures of the prenatal dose are more controversial because the developing fetal brain is the exclusive target for the toxic action of MeHg. This dose can only be estimated by indirect measures (usually described as "biological monitoring") and the rationale for the choice of the appropriate biologic marker must come from an understanding of the disposition of mercury in the body, and more specifically in the maternal–fetal unit (4,11). The majority of studies of prenatal exposure to MeHg have used hair and blood; however, there is ongoing discussion as to which medium will be more likely to give a better index of levels of MeHg in the fetal brain. The real value of hair as an appropriate biologic indicator comes from an examination of the degree of correlation with the levels of mercury in the target tissue, the brain (Fig. 50.5). The relationships between levels of mercury in maternal hair versus levels in four anatomic regions of autopsy brains from infants dying shortly after birth have demonstrated a linear association, with correlation coefficients in the range of 0.8. Given the variance that inevitably arises from measures of autopsy tissues as well as the long distance transport and lengthy storage of some of these samples, these correlations are quite striking, and clearly support the use of maternal hair as an indicator of fetal brain levels (11,44).

A close correlation of total mercury in hair and blood has been shown in individuals exposed to MeHg (45) and in the human scalp/nude mouse model (Fig. 50.4). However, the concentration ratio of mercury in hair to mercury in blood differs from one individual to another. An average ratio of 250:1 has been assumed by expert committees (1) but the range of values extends from less than 200 to more than 300 and the ratio may be age dependent (46). These differences in ratios have been claimed to undermine hair as an appropriate biologic indicator compared with direct measure of mercury in blood samples (47). In fact, the opposite conclusion should be drawn. As discussed earlier, hair accumulates

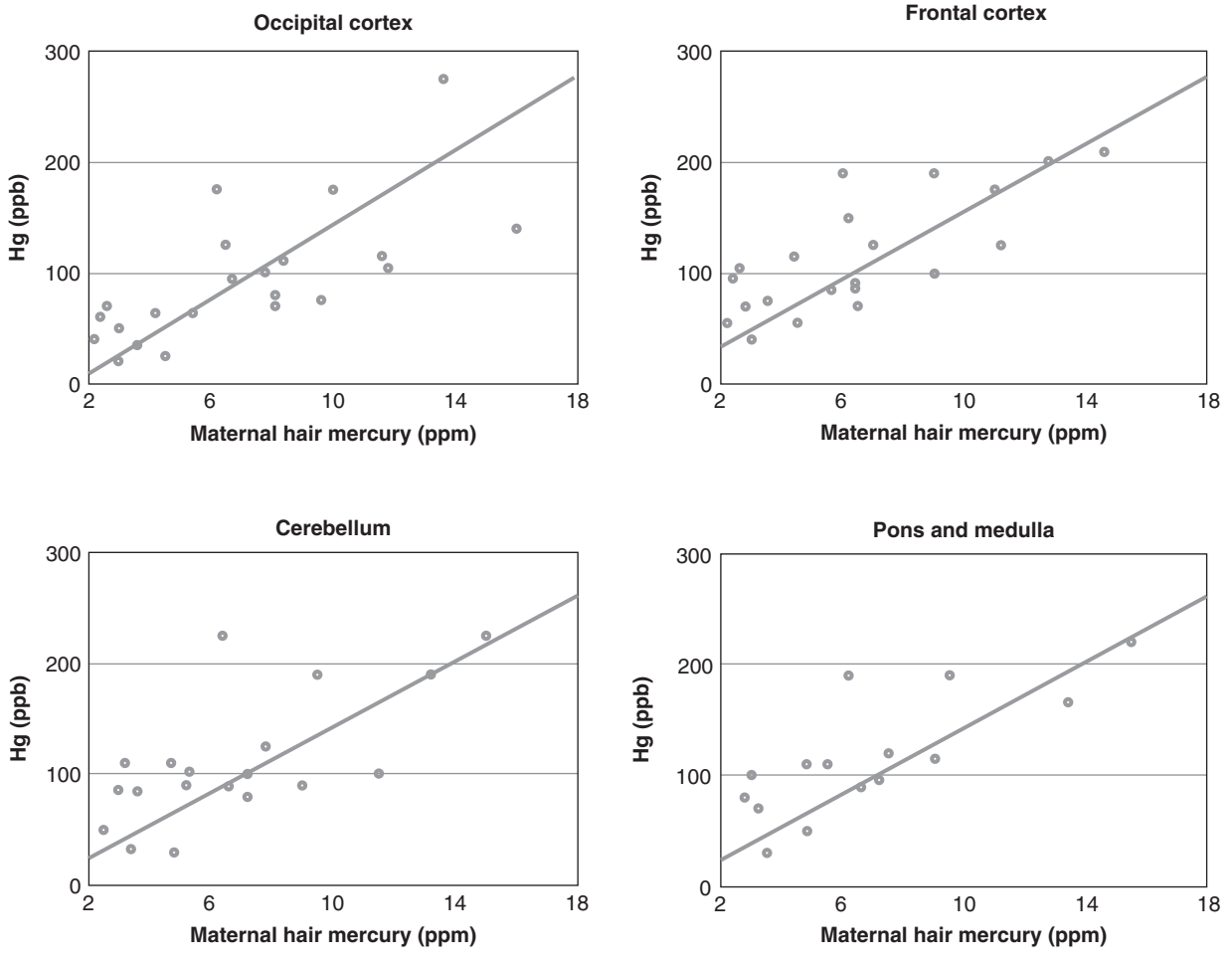

FIGURE 50.5 Mercury concentrations in four anatomical areas of the infant brain plotted against the concentration in maternal hair. The infants died soon after birth from various natural causes unrelated to their methyl mercury exposure. The maternal hair mercury concentrations were measured in the first centimeter next to the scalp. Hair was collected at the time of the infants' death.

the same transportable species of MeHg as crosses the blood–brain barrier, namely, the MeHg–cysteine complex. This transport species must account for only a minute fraction of total mercury in blood. Some 80–90% of MeHg in blood is bound to hemoglobin in red blood cells. Plasma MeHg is mainly bound to mercaptalbumin (48,49), leaving only a tiny fraction bound to small molecules with thiols, such as L-cysteine. Changes in this small fraction of MeHg–cysteine complex in plasma are the most plausible explanation for differences in hair-to-blood ratios of total mercury seen in different individuals. In addition, blood levels of total mercury will also be affected by hematocrit as most mercury is located in the red blood cells. For example, when total mercury in cord blood is used as a measure of the prenatal dose of MeHg, hematocrit in this case, depends appreciably on the method of collection (50,51). These considerations suggest that total mercury levels in hair are the better indicator of brain levels than levels of total mercury in whole blood.

CONCLUSIONS

Maternal scalp hair mercury levels have been used as an index of mercury levels in both maternal and fetal tissues in most epidemiologic studies. The mechanism of entry of MeHg into the hair follicle supports the widespread use of human scalp hair as the biologic indicator of choice for human exposure to MeHg. Levels of mercury in maternal scalp hair at the time of delivery have been shown to correlate closely with levels of mercury in autopsy brain tissue of newborn and neonatal infants (11,44). Such findings are consistent with the proposed mechanism that hair levels of mercury reflect the concentration in blood of the species transported into the brain, namely, the MeHg–cysteine complex.

In addition, it was demonstrated that the human scalp/nude mouse model can accurately record the level of MeHg exposure and that this model can serve as a valuable research tool to study the mechanisms of MeHg incorporation into human hair. This unique system can also serve in many more emerging areas of investigative dermatology, especially in hair research, and may help to develop better understanding of hair follicle biology.

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51 Use of modified forearm-controlled application test to evaluate skin irritation of lotion formulations*

Miranda A. Farage and Klaus-Peter Wilhelm

INTRODUCTION

Skin effects of paper products are a combination of the inherent chemical irritation of the materials that make up the product and some degree of mechanical irritation due to friction during use. Safety evaluation programs on paper products usually involve test methods designed to detect potential irritation under exaggerated conditions. Typically, the evaluation begins with patch testing to confirm an absence of frank irritation, followed by a simulated use test. Often, a clinical study will be conducted as a final step in the evaluation process.

Modern facial tissue products are mild to skin. In fact, the current emphasis of manufacturers is to develop products that will provide skin benefits, rather than just an absence of negative effects. For example, a number of products are currently marketed that contain a lotion coating to aid in the healing or prevention of irritation that may occur with repeated, frequent use of the tissue product by cold or allergy sufferers.

A number of short-term screening test protocols have been developed to evaluate the potential benefits of lotion or moisturizing products with regard to the effects on normal skin, the ability to accelerate healing in irritated skin, and the prevention of irritation. Many of these test systems use the flexor surface of the forearm as a test site. Serup (2) evaluated intact skin using a single application of moisturizers to the flexor surface of the forearm in the absence of any pretreatment to induce irritation. The responses of the treated skin were assessed by measurements of epidermal hydration, scale pattern, and skin surface lipids. In a study conducted by Jemec and Na (3), volunteers used moisturizers on the volar surface of the forearm for 21 days; once a day on one arm and twice a day on the other arm. The mechanical properties of the skin were measured, as was skin capacitance.

In order to evaluate the effects on irritated skin, a number of protocols have used skin irritated by pretreatment with sodium lauryl sulfate (SLS), which is widely used as a model irritant in studies on skin effects (4). Blanken et al. (5) used a protocol that interspersed 45-minute applications of a low concentration of SLS (0.5%) with applications of fatty preparations over a period of two weeks, and evaluated the efficacy of the preparations using measures of skin vapor loss. De Paepe et al. (6) developed a model to support the efficacy claims of body lotions. The model consisted of applying body lotion formulations to the skin of the flexor surface of the forearm twice a day for two weeks to normal skin and skin that had been pretreated via a 24-hour patch with 1.25% SLS. Responses were evaluated using transepidermal water loss (TEWL) and capacitance measurements.

Held et al. (7) used a 24-hour patch pretreatment with 0.5% SLS to induce mild irritation on the upper arms and forearms to compare the efficacy of several different moisturizer formulations. These investigators used a similar model to evaluate the effects of moisturizers on the susceptibility to irritants (8). Test subjects applied moisturizers to the upper arms and forearms three times daily for five days. This was followed by a 24-hour patch of 0.25% SLS. Reactions were evaluated by a variety of methods, including visual scoring for erythema and TEWL. Zhai et al. (9) used a single pretreatment of test sites on the flexor aspect of the forearms with a dimethicone protectant lotion or vehicle control. This pretreament was followed by a 24-hour occluded patch with 0.5% SLS. The effectiveness of the protectant lotion was determined by visual scoring, TEWL, skin color (a* value), and cutaneous blood flow volume.

In cold sufferers, the irritation around the nostrils is a combination of effects, including the mucous running from the nose, the inherent irritant properties of the tissue components (chemical irritation), and mechanical irritation from friction resulting from frequent and repeated rubbing of the skin site with the tissue. Adding lotion to the tissue adds another source of potential irritating materials that may further contribute to the overall irritation, especially under conditions of application to damaged skin. We were interested in developing a model that incorporated all the elements that contribute to the complicated conditions experienced by an individual suffering from a cold or allergies, such as chemical irritation of the tissue and lotion components, and mechanical irritation, to evaluate the potential skin benefits of various lotion formulations. We chose to adapt a test system that has been used to evaluate personal cleansing products and baby wipes, and is a modification of the forearm-controlled application technique (FCAT) (10–12).

The objective of these studies was to evaluate the efficacy of different lotion formulations on facial tissues in preventing irritation, or aiding in the healing of irritation. We added a patch test with SLS to the modified FCAT protocol, to simulate an underlying irritation, such as that which may exist in a cold sufferer. Test sites were treated by repeated wiping with facial tissues to simulate the

*This chapter is adapted from (1), with kind permission from Blackwell-Wiley Publishing.

product use by a cold sufferer. Visual assessment of erythema and dryness was used to determine if skin benefit(s) were detected.

METHODOLOGY

Test Protocols

The basic test protocol and protocol variations are presented in Figure 51.1. Within this basic design, several modifications were used, including variations in the concentration of SLS used, the number of total tissue wipes, and the number of days of grading. Specific details of the protocol are given in the appropriate figure or table legend.

In each experiment, two to three test sites were identified and demarcated on each volar surface of the forearm. Test sites measured 4 × 4 cm, and were 4 cm apart. Each site was treated with a different test product. The products were randomized, and the technician conducting the test was not aware of the test product identity.

For the tissue wipes, each tissue was folded up to five times, and wiped 10 times in a back and forth movement (20 passes). The test tissue was then refolded and the wiping repeated with a fresh area of the tissue. New tissues were used, as needed until the total number of back and forth wipes was completed. The SLS was patched using a Webril® patch (Kendall Healthcare, Mansfield, Massachusetts, USA) covered by an occlusive, hypoallergenic tape (Blenderm®, 3M Healthcare, St. Paul, Minnesota, USA).

Grading of the test sites was done at intervals shown in Figure 51.1. When SLS patching was conducted, the patches were removed 30–60 minutes before grading. In all studies, visual scoring was conducted by expert graders under a 100 W incandescent daylight bulb. Erythema and dryness were graded on separate scales according to a previously described scale of 0–6. On both scales, "0" indicated perfect skin and "6" indicated severe erythema or severe scaling with bleeding cracks for erythema and dryness, respectively (12). Visual assessment of skin reactions has been used for decades to evaluate irritant reactions. A review of the use of visual grading scales supports the overall reliability of visual assessment for a wide variety of test protocols (13). In an earlier publication on the modified FCAT protocol (12), a direct comparison was made of visual and instrumental scoring methods. The results of the visual scoring method were very similar to those of the instrumental scoring methods.

In some studies, reactions were also evaluated using measurement of TEWL (DermaLab® Evaporimeter Cortex Technology, Denmark). Prior to this measurement, subjects remained in a humidity- and temperature-controlled room for approximately 20 minutes to equilibrate with the ambient conditions.

Materials Tested

Samples evaluated in the program were either currently or recently marketed facial tissue products (with or without lotion coating), or the currently marketed facial tissue substrate coated with various test lotions. The components of the various lotion formulations tested are given in Table 51.1. All lotions were extruded onto the same tissue substrate at a concentration of 5 pounds of lotion per 3000 sq ft (or 0.815 mg/cm^2) of tissue substrate unless otherwise indicated.

Test Subjects

The protocol was approved by the test facility's Institutional Review Board. Participants in all the studies were 18- to 55-year-old healthy female adult volunteers who had signed an informed consent. Subjects were excluded from participation if (*i*) they were currently participating in any other clinical study; (*ii*) they had participated in any type of research study involving the forearms within the previous 21 days; (*iii*) they had allergies to soap, detergent, perfume, cosmetics, and/or toiletries; (*iv*) they were taking anti-inflammatory corticosteriods or other medications that may interfere with test results; (*v*) they had had eczema or psoriasis within the past six months; (*vi*) they had been diagnosed with skin cancer within the previous 12 months; (*vii*) they were pregnant or lactating; or (*viii*) they had cuts, scratches, rashes, or any condition on their inner forearms that may prevent a clear assessment of their skin.

Basic protocol

Day 0		Day 1			Day 2	Day 3	Day 4
Grade	Patch	Grade	Tissue wipe	Grade	Grade	Grade	Grade
Baseline	24-h SLS	Post irritation		Post tissue wipe	24 hr post tissue wipe	48 hr post tissue wipe	72 hr post tissue wipe

Variation 1

Day 0	Day 1				Day 2	Day 3
	Grade	Tissue wipe	Grade	Patch	Grade	Grade
	Baseline		Post tissue wipe	24	24 hr post irriation	48 hr post irritation

Variation 2

Day 0		Day 1			Day 2			Day 3	Day 4
Grade	Patch	Grade	Tissue wipe	Grade	Grade	Short tissue wipe	Grade	Grade	Grade
Baseline	24-h SLS	Post irritation		Post tissue wipe			Post 2nd tissue wipe	48 hr post 2nd tissue wipe	72 hr post 2nd tissue wipe

FIGURE 51.1 Basic protocol design and variations. Grading is via visual scoring for erythema and dryness. Transepidermal water loss was recorded for one experiment. *Abbreviation*: SLS, sodium lauryl sulfate. From Ref. 1.

TABLE 51.1
Ingredients in Lotion Formulations Tested

	Currently Marketed Lotions			Experimental Lotions			
Lotion code	*P-CSA*	*Q-SA*[a]	*B-SA*	*B-CSA*	*A-SA*	*A-CSA*	*C*
Formula base	Mineral oil	Mineral oil and isopropyl palmitate	Mineral oil and petrolatum	Mineral oil and petrolatum	Petrolatum	Petrolatum	—
Fatty alcohol	Cetearyl alcohol	Stearyl alcohol	Stearyl alcohol	Cetearyl alcohol	Stearyl alcohol	Cetearyl alcohol	—
Other components	Paraffin wax, steareth-2, aloe, and vitamin E	Cerasin, calendula oil, dimethicone	—	—	Fatty acid sucrose esters	Fatty acid sucrose esters	Fatty acid sucrose esters
Lotion concentration on tissue substrate (mg/cm^2)	0.815 (unless otherwise stated)	Unknown	0.815	0.815	0.815	0.815	0.815
Silicone version	Lotion was extruded onto tissue substrate with either 3000 or 4500 ppm silicone	A version with silicone is also available	—	—	Lotion was extruded onto tissue substrate with 3000 ppm silicone	—	—

[a]Q-SA is a currently marketed competitor's product. Some of the product details are unknown.
Source: From Ref. 1

Eligible subjects were given a sensitive skin care cleansing bar for all bathing and showering needs to be used beginning with their enrollment into the study and until their participation in the study was complete. They were instructed to avoid scrubbing the inner forearm areas and allow the soap and water to flow over the areas without washing. In addition, they were required to refrain from using lotions, creams, ointments, oils, and/or moisturizers on the forearm areas. Study groups consisted of 13–19 subjects.

Analyses of Data

The mean score for erythema [± standard error (SE)] for the panel was determined at each scoring time point. The postwipe average was calculated using the average of all postwipe visit scores for each subject, then calculating the mean (± SE) over all subjects. If there were missing visits for a subject, that subject was not included in the calculation of the postwipe average.

In those experiments where SLS patching occurred prior to treatment with the lotion, the results are presented as the change in group mean. The change in group mean was calculated by determining the change from post-SLS baseline (i.e., the postirritation score) for each subject, then calculating the average over all the subjects. In some cases, not all test subjects completed the entire test. In these instances, the scores recorded for the dropped subjects were removed from the calculation of the change in group mean for that time point.

All statistical analyses are described in the table and figure legends.

RESULTS

Establishing the Basic Protocol

The model was examined by assessing the reactions in the presence and absence of pretreatment by patching with SLS, followed by wiping with several lotion formulations. Typical results are shown in Table 51.2. Using lotion P-CSA in the absence of pretreatment with SLS, there is virtually no evidence of dryness or erythema resulting from wiping with a lotion-coated tissue. However, patching with SLS establishes an underlying irritation as shown by an increase in both dryness and erythema.

Effect of Lotion on Prevention of Irritation

Sites were treated by wiping with the tissues prior to patching with SLS (protocol variation 1 in Fig. 51.1) to evaluate the effectiveness of two different lotion formulations on preventing irritation. Results of the scoring for dryness and erythema are shown in Figure 51.2. For all four treatments, scoring immediately after the tissue wipes (post-tissue wipe grade) showed no significant increase in either dryness or erythema compared with baseline values. Scores at both time points after patching with SLS (24 and 48-hour postirritation) were significantly elevated for all four treatments. However, there were no significant differences between the reactions at sites treated with the lotioned products and their nonlotioned controls, although the dryness scores tended to be lower when the tissue contained lotion.

Effect of Lotion on Healing Irritation

We examined the effect of various lotion products on the speed of recovery of skin irritated by patching with SLS. The same lotion formulations evaluated in the previous experiment were evaluated in this protocol, using the basic protocol (as shown in Figure 51.1). Results from lotion P-CSA are shown in Table 51.3. The presence of P-CSA lotion on the tissues had a significant effect on dryness. Immediately following the tissue wipe, the lotioned tissue caused a substantial reduction in dryness from a mean group score of 1.06–0.39; a change in group mean score of −0.67. The nonlotioned tissue produced a much more modest reduction in dryness, with a change in the group mean score of −0.18. The mean scores for dryness at the lotion-treated sites were significantly lower than

the control sites at two of the three time points. The erythema reactions for both the lotioned and nonlotioned tissues increased slightly immediately following the tissue wiping procedure, and remained elevated for the duration of the test. Determining the average change in dryness and erythema for all post-tissue wipe scores (i.e., post-tissue wipe average) provides a means to rank order test materials for overall benefits. In this instance, the test tissue with lotion P-CSA produced a greater reduction in average dryness scores than the test tissue without lotion (−0.48 *vs* −0.06). The average change in erythema scores was similar for the two test products (0.36 and 0.32).

Figure 51.3 presents the results for both test products (lotion P-CSA and lotion Q-SA) from this same experiment as the change in group mean scores (dryness and erythema) from those scores recorded after patching with 0.25% SLS. The presence of lotion P-CSA directionally reduced dryness on test sites pretreated by SLS patching, with the results reaching significance at two scoring time points (Fig. 51.3A). The presence of lotion Q-SA did not produce significant improvements in dryness compared with the nonlotioned control. Lotion P-CSA produced directionally less erythema than lotion Q-SA, although the results did not reach significance (Fig. 51.3B). As indicated by the postwipe average scores, the test tissue with P-CSA lotion produced the greatest average improvement in dryness scores of the four test products. The test tissue with Q-SA produced the highest average level of erythema.

Table 51.2

Effect of P-CSA Lotion in Preventing SLS-Induced Irritation

Scoring Time Point	*n*	Dryness (Mean ± SE)			Erythema (Mean ± SE)		
Tissues with P-CSA lotion (no SLS pretreatment)							
Baseline	18	0.00	±	0.00	0.00	±	0.00
Post-tissue wipe	18	0.00	±	0.00	0.11[a]	±	0.05
24-hr Post-tissue wipe	18	0.11	±	0.08	0.00	±	0.00
48-hr Post-tissue wipe	18	0.06	±	0.06	0.00	±	0.00
72-hr Post-tissue wipe	18	0.11	±	0.08	0.00	±	0.00
Tissues with P-CSA lotion (pretreatment with SLS)							
Baseline	16		NA		0.00	±	0.00
Postirritation	16	1.62	±	0.24	1.09	±	0.11
Post-tissue wipe	16	0.31[b]	±	0.15	1.44[b]	±	0.13
24-hr Post-tissue wipe	16	0.44[b]	±	0.13	1.12	±	0.15
48-hr Post-tissue wipe	16	0.19[b]	±	0.10	0.91	±	0.15
72-hr Post-tissue wipe	16	0.19[b]	±	0.10	0.59[b]	±	0.13

Note: The basic test protocol was used, as shown in Figure 51.1. Test sites on the flexor surfaces of the forearms were pretreated by 24-hr patch with 0.1% SLS. After patch removal, test sites were wiped with the test tissues using a total of 120 wipes (240 passes).

[a]Significantly different from baseline score ($P < 0.05$).

[b]Significantly different from postirritation score ($P < 0.05$).

Abbreviations: NA, not available; SLS, sodium lauryl sulfate; P-CSA, formulation with mineral oil base with cetearyl alcohol.

Source: From 1.

Evaluation of Formula Options

The effects of two lotion formulations with and without silicone on dryness and erythema were compared (Fig. 51.4). One of these formulations (P-CSA) was a mineral oil base with cetearyl alcohol. The other (A-SA) was a petrolatum base with stearyl alcohol. In the absence of silicone, both formulas caused a reduction in dryness from the post-tissue wipe score (Fig. 51.4A). In the case of lotion P-CSA, this reduction was substantial; however, it did not reach statistical significance. The addition of 3000 ppm silicone

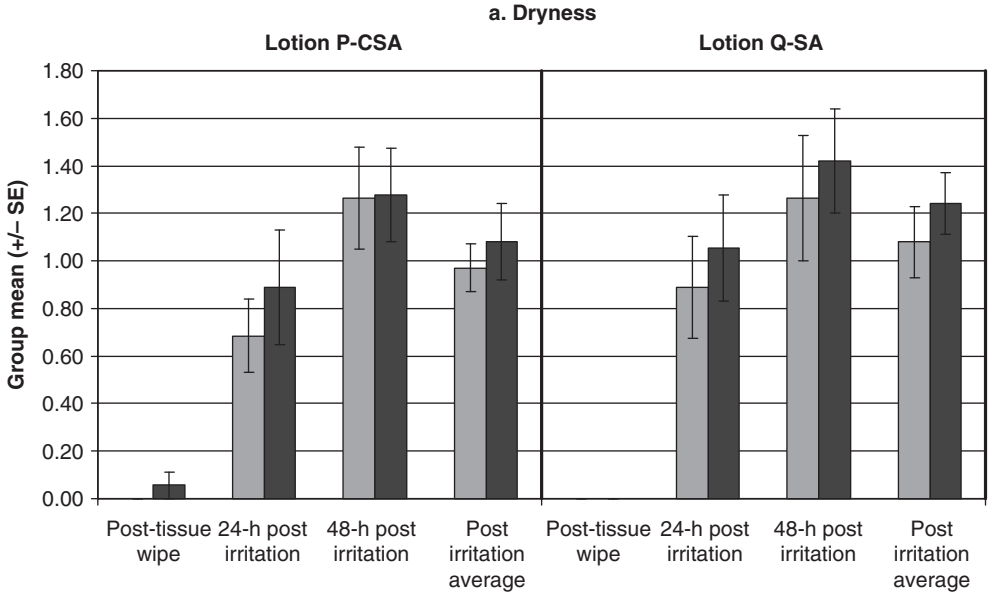


FIGURE 51.2 Effect of pretreatment with lotion. Protocol variation 1 was used, as shown in Figure 51.1. Test sites on the flexor surfaces of the forearms of 19 subjects were wiped with the test tissues on day 1 using a total of 200 wipes (400 passes). This was followed by a 24-hr occlusive patch with 0.25% sodium lauryl sulfate. The group mean scores for dryness (**A**) and erythema (**B**) were determined for each scoring time point. Postirritation average treatment comparisons were performed using analysis of variance. All other treatment comparisons were performed using the stratified Cochran–Mantel–Haentzel test. For lotion P-CSA, the concentration of lotion on the tissues was 0.668 mg/cm^2. Since Q-SA is a currently marketed competitor's product, the lotion concentration is unknown. [a]Control for P-CSA (without lotion) different from Q-SA (with lotion) ($P < 0.05$).
Abbreviations: P-CSA, formulation with mineral oil base with cetearyl alcohol; Q-SA, the competitive product containing dimethicone. From Ref. 1.

TABLE 51.3
Effects of Pretreatment with P-CSA Lotion on Healing SLS-Induced Irritation

		Dryness						Erythema					
		Group Mean Score			Change from Post Irritation Score			Group Mean Score			Change from Post Irritation Score		
Scoring Time Point	*n*	(Mean	±	SE)	(Mean	±	SE)	(Mean	±	SE)	(Mean	±	SE)
Tissues with P-CSA lotion													
Baseline	18	0.06	±	0.06				0.00	±	0.00			
Post irritation	18	1.06	±	0.19				0.92	±	0.10			
Post-tissue wipe	18	0.39[a]	±	0.12	−0.67	±	0.21	1.25	±	0.13	0.33	±	0.08
24-hr Post-tissue wipe	18	0.89	±	0.11	−0.17	±	0.22	1.33	±	0.17	0.42	±	0.16
48-hr Post-tissue wipe	18	0.44[a]	±	0.12	−0.61	±	0.26	1.25	±	0.22	0.33	±	0.21
Post-tissue wipe average	18	0.57[a]	±	0.08	−0.48	±	0.22	1.28	±	0.14	0.36	±	0.12
Control (tissues with no lotion)													
Baseline	17	0.11	±	0.08				0.00	±	0.00			
Postirritation	17	0.94	±	0.10				0.85	±	0.09			
Post-tissue wipe	17	0.76	±	0.11	−0.18	±	0.15	1.15	±	0.12	0.29	±	0.06
24-hr Post-tissue wipe	17	1.12	±	0.12	0.18	±	0.18	1.29	±	0.08	0.44	±	0.12
48-hr Post-tissue wipe	17	0.76	±	0.11	−0.18	±	0.15	1.09	±	0.18	0.24	±	0.17
Post-tissue wipe average	17	0.88	±	0.06	−0.06	±	0.13	1.18	±	0.10	0.32	±	0.09

Note: The basic test protocol was used, as shown in Figure 51.1, except scoring was not conducted at 72-hr post-tissue wipe. Test sites on the flexor surfaces of the forearms were pretreated by 24-hr patch with 0.25% SLS. After patch removal, test sites were wiped with the test tissues (tissues with 0.668 mg/cm^2 lotion P-CSA) using a total of 200 wipes (400 passes). The change from the postirritation score was determined for each subject, then the average over all the subjects was calculated. The post-tissue wipe average was calculated using the average of all postwipe scores for each subject, then calculating the average over all the subjects. Treatment comparisons for erythema for post-tissue wipe time points were performed using analysis of covariance. Post-tissue wipe average treatment comparisons were performed using analysis of variance. All other comparisons were performed using stratified Cochran–Mantel–Haentzel test.

[a]Significantly lower than control ($P < 0.05$).

Abbreviations: NA, not available; SLS, sodium lauryl sulfate; P-CSA, formulation with mineral oil base with cetearyl alcohol.

Source: From Ref. 1.

resulted in even more marked improvements in dryness scores for both the lotion formulations. Erythema scores in the presence of silicone tended to be greater (Fig. 51.4B). As indicated by the post-wipe averages, lotion P-CSA with 3000 ppm silicone produced the greatest overall reduction in dryness. However, this formula combination also produced the greatest overall increase in erythema.

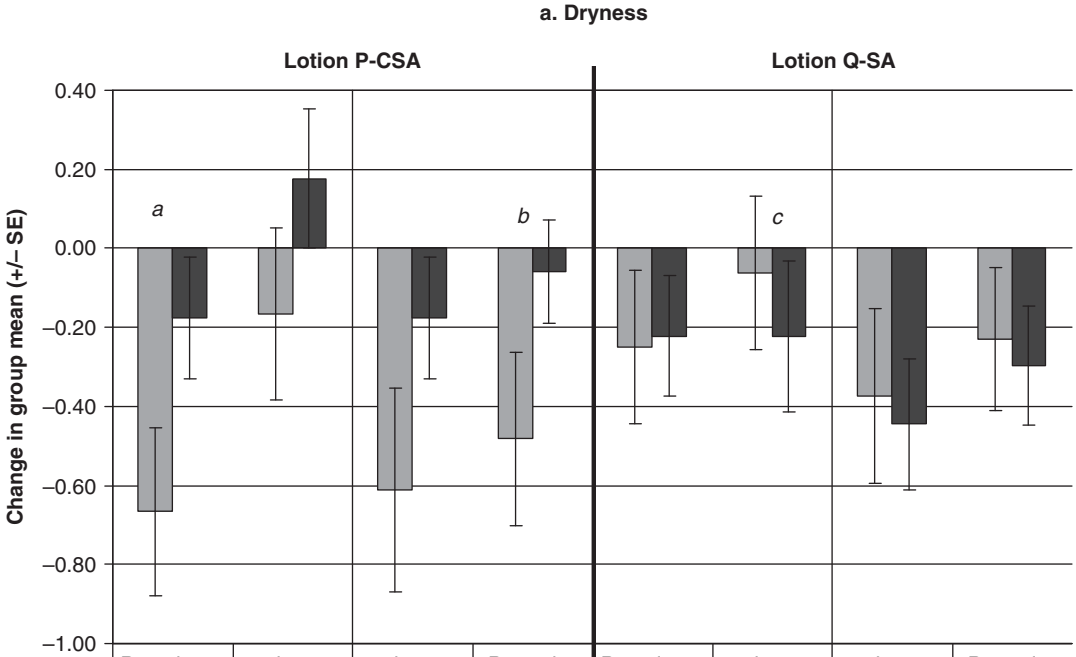
The effects of different levels of silicone in the presence of the same lotion formulation were also evaluated. Figure 51.5 shows a comparison between test products in which lotion P-CSA was applied to tissue substrate containing either no silicone, 3000 ppm silicone, or 4500 ppm silicone. The presence of 3000 ppm silicone produced slightly greater reductions in dryness at some time points than the lotion in the absence of silicone (Fig. 51.5A). Increasing the silicone to 4500 ppm reversed this trend, with lesser reductions in dryness. The levels of erythema were slightly improved in both silicone test groups compared with tissues with no silicone (Fig. 51.5B). In this study, measures of TEWL were also included in the evaluation (Fig. 51.5C). The TEWL results indicated slight improvements with either 3000 or 4500 ppm silicone compared with no silicone.

An experiment was conducted to compare the relative skin effects of cetearyl alcohol and stearyl alcohol. Two different lotion base formulations were tested: one with a petrolatum base and one with a mineral oil/petrolatum base. Each formulation was prepared using cetearyl alcohol and stearyl alcohol. All other components of the formulations were identical. As seen in Figure 51.6, the stearyl alcohol gave results very similar to those of cetearyl alcohol for both dryness and erythema.

Four formulations with different emollients were compared. These formulations consisted of a mineral oil base (P-CSA), a mineral oil/petrolatum base (B-CSA), a petrolatum base (A-CSA), and a proprietary mixture of fatty acid sucrose esters (C). Results (shown in Fig. 51.7) indicated that all four formulations had comparable benefits with regard to the effects on dryness (Fig. 51.7A). However, the test sites treated with proprietary formulation (C) exhibited less erythema than the other formulations at the final two scoring time points (48 and 72-hour post-tissue wipe in Fig. 51.7B). The change in the 48-hour score was significantly different from the other three formulations. The change in the 72-hour score was significantly different from formulation P-CSA.

DISCUSSION

Consumers suffering from colds and allergies are particularly vulnerable to the irritant effects of repeated, frequent use of tissue products. These effects include the inherent chemical irritation of the tissue substrate and lotion materials, and the mechanical irritation from friction. We were interested in developing a model that included these elements as a way to quickly screen candidate materials for lotion coatings, and as a means of claims support. A number of investigators have used applications of materials to the flexor surface of the forearm, with and without pretreatment



	Post-tissue wipe	24-h post-tissue wipe	48-h post-tissue wipe	Post wipe average	Post-tissue wipe	24-h post-tissue wipe	48-h post-tissue wipe	Post wipe average
With lotion	–0.67	–0.17	–0.61	–0.48	–0.25	–0.06	–0.38	–0.23
Control (without lotion)	–0.18	0.18	–0.18	–0.06	–0.22	–0.22	–0.44	–0.30

FIGURE 51.3 Effect of lotion treatment on sodium lauryl sulfate–patched skin. The basic test protocol was used, as shown in Figure 51.1. Experimental details are described in Table 51.3. The postwipe average was calculated using the average of all postwipe visits for each subject, then calculating the average over all the subjects. The changes from the postwipe scores are given. Treatment comparisons for the change in erythema at 24- and 48-hr post-tissue wipe, and the change in postwipe average were performed using analysis of variance on ranks. All other treatment comparisons were performed using the stratified Cochran–Mantel–Haentzel test. The concentration of P-CSA lotion on the tissues was 0.668 mg/cm^2. [a]P-CSA (with lotion) different from control Q-SA (without lotion) ($P < 0.05$). [b]P-CSA (with lotion) different from control P-CSA (without lotion) ($P < 0.05$). [c]Control Q-SA (without lotion) different from control P-CSA (without lotion) ($P < 0.05$). [d]Controls P-CSA and Q-SA (without lotion) different from Q-SA (with lotion) ($P < 0.05$).
Abbreviations: P-CSA, formulation with mineral oil base with cetearyl alcohol; Q-SA, the competitive product containing dimethicone. From Ref. 1.

with SLS, as a means of evaluating the effectiveness of lotions. We added an element to this approach by incorporating repeated wipes with the lotion-coated tissues to include a mechanical irritation component.

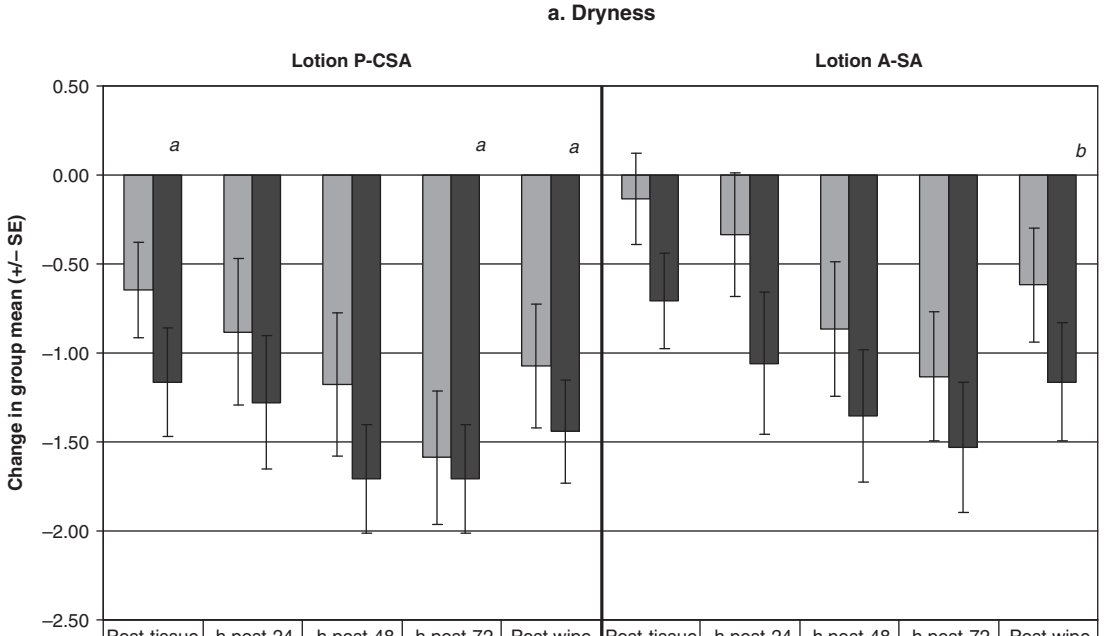
The results shown in Table 51.2 confirm that treatment with the lotion-coated tissue in the absence of SLS pretreatment results in no visible irritation, as indicated by virtually no increase in either dryness of erythema scores. Therefore, pretreatment with SLS to establish a background level irritation was essential to enable the detection of differences in irritant effects.

Two lotion formulations were tested for protective effects against irritation by SLS, P-CSA, and Q-SA, as shown in Figure 51.2. As expected, such short-term treatments with lotion were not particularly effective in preventing irritation, although dryness scores tended to be slightly lower when lotion was present on the tissue. Interestingly, Zhai et al. (9) reported a significant reduction in both visual scores and TEWL when a single application of dimethicone was used prior to patching with 0.5% SLS. In our study, the competitive product (Q-SA) contained dimethicone; however, there was no indication of a protective effect.

These same lotions were tested for healing effects after SLS pretreatment (Fig. 51.3). Lotion P-CSA was effective in reducing dryness, compared with the tissue without lotion, but was not effective in treating erythema. Lotion Q-SA did not appear to have an effect in reducing dryness compared with the tissue without lotion, and the presence of the Q-SA lotion actually increased erythema (significantly, at post-tissue wipe scoring time point). Both of these lotion formulations contain mineral oil. Lotion Q-SA also contains isopropyl palmitate and dimethicone. The lotions also differ in the fatty alcohol, with P-CSA containing cetearyl alcohol and Q-SA containing stearyl alcohol.

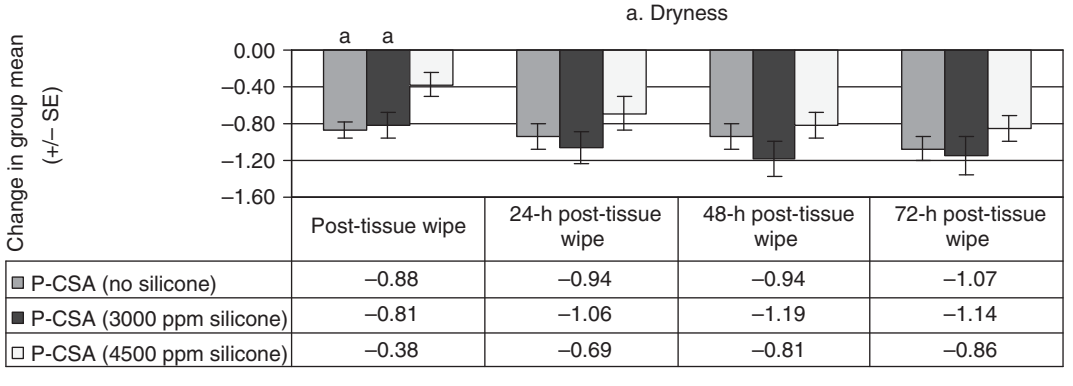
In the study shown in Figure 51.4, the effectiveness of a silicone layer on the tissue in healing SLS-induced irritation was evaluated for two formulations: P-CSA (mineral oil base with cetearyl alcohol) and A-SA (petrolatum base with stearyl alcohol). For formula P-CSA, the presence of silicone improved the dryness scores, as shown by a greater reduction in scores at individual time points than the same lotion without silicone, and a greater postbaseline average reduction (Fig. 51.4A). For lotion A-SA, silicone had a similar, although less pronounced, effect. However, the presence of silicone also increased the erythema scores slightly over lotion without silicone.

The improvement in dryness scores and increase in erythema scores when silicone was added to P-CSA lotion was not repeated in an experiment comparing different levels of silicone (Fig. 51.5). In this experiment, the dryness scores without silicone and with 3000 ppm silicone were much more similar than the results shown in Figure 51.4. An increase in silicone to 4500 ppm appeared to

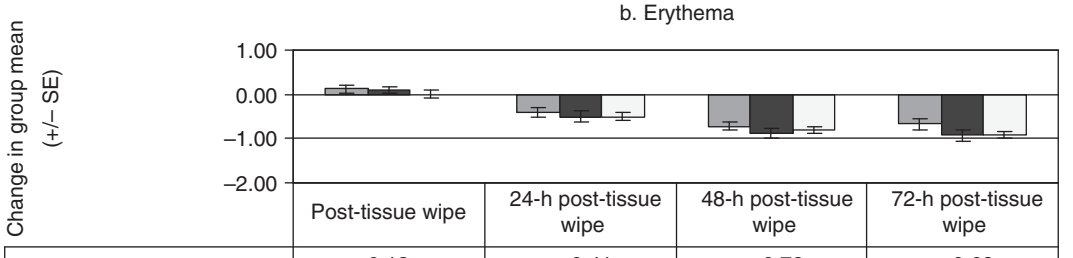


	Post-tissue wipe	h post-24 tissue wipe	-h post-48 tissue wipe	-h post-72 tissue wipe	Post wipe average	Post-tissue wipe	h post-24 tissue wipe	-h post-48 tissue wipe	-h post-72 tissue wipe	Post wipe average
Without silicone	0.65–	0.88–	1.18–	1.59–	1.07–	0.13–	0.33–	0.87–	1.13–	0.62–
With 3000 ppm silicone	1.17–	1.28–	1.71–	1.71–	1.44–	0.71–	1.06–	1.35–	1.53–	1.16–

FIGURE 51.4 Effect of silicone on dryness and erythema. The basic test protocol was used, as shown in Figure 51.1, with the same experimental details as described in Figure 51.3 and Table 51.3. The postwipe average and the changes from the postwipe scores were determined as described above. Erythema post-tissue wipe average comparisons were performed using ANOVA. All other treatment comparisons were performed using ANOVA on ranks. The concentration of the lotions on the tissues was 0.668 mg/cm^2 for P-CSA, and 0.815 mg/cm^2 for A-SA. For both formulations, the amount of silicone on the tissue substrate was 3000 ppm. [a]P-CSA (with silicone) different from A-SA (without silicone) ($P < 0.05$). [b]A-SA (with silicone) different from P-CSA (without silicone) ($P < 0.05$). *Abbreviations*: ANOVA, analysis of variance; A-SA, petrolatum base with stearyl alcohol; P-CSA, formulation with mineral oil base with cetearyl alcohol; Q-SA, the competitive product containing dimethicone. *Source*: Adapted from Ref. 1.



	Post-tissue wipe	24-h post-tissue wipe	48-h post-tissue wipe	72-h post-tissue wipe
P-CSA (no silicone)	–0.88	–0.94	–0.94	–1.07
P-CSA (3000 ppm silicone)	–0.81	–1.06	–1.19	–1.14
P-CSA (4500 ppm silicone)	–0.38	–0.69	–0.81	–0.86



	Post-tissue wipe	24-h post-tissue wipe	48-h post-tissue wipe	72-h post-tissue wipe
P-CSA (no silicone)	0.13	–0.41	–0.72	–0.68
P-CSA (3000 ppm silicone)	0.09	–0.50	–0.88	–0.93
P-CSA (4500 ppm silicone)	0.00	–0.50	–0.81	–0.93

FIGURE 51.5 Effects of different levels of silicone. Protocol variation 2 was used, as shown in Figure 51.1. Test sites on the flexor surfaces of the forearms of 13–16 subjects were pretreated by 24-h patch with 0.1% sodium lauryl sulfate. After patch removal, test sites were wiped with the test tissues using a total of 120 wipes (240 passes). On the following day, the test sites were wiped with the test tissues for an additional 10 wipes (20 passes). The changes from the postwipe scores are shown. Comparisons for dryness and erythema were performed using stratified Cochran–Mantel–Haentzel test. Comparisons for transepidermal water loss were performed using analysis of covariance. [a]P-CSA (without and 3000 ppm silicone) different from P-CSA (4500 ppm silicone) ($P < 0.05$). *Abbreviation*: P-CSA, formulation with mineral oil base with cetearyl alcohol. From Ref. 1.

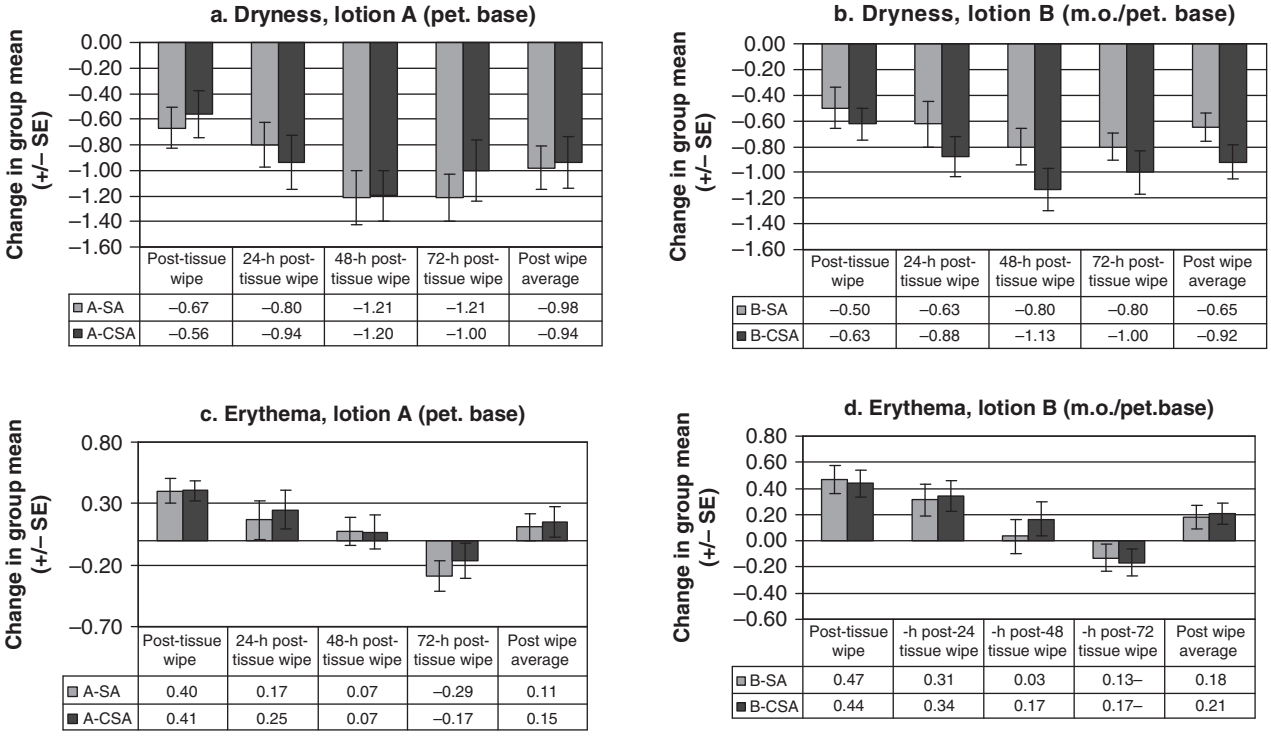


FIGURE 51.6 Comparison of stearyl alcohol and cetearyl alcohol in two different base formulations. The basic test protocol was used, as shown in Figure 51.1. Test sites on the flexor surfaces of the forearms of 15–16 subjects were pretreated by 24-hr patch with 0.1% sodium lauryl sulfate. After patch removal, test sites were wiped with the test tissues using a total of 120 wipes (240 passes). Tissues were coated with either base formula (**A**) containing either stearyl or cetearyl alcohol (A-SA and A-CSA, respectively), or base formula (**B**) containing either stearyl or cetearyl alcohol (B-SA and B-CSA, respectively). The changes from the postwipe scores are shown. Comparisons were performed using analysis of variance. Differences between treatments did not reach significance. *Abbreviations*: A-SA, petrolatum base with stearyl alcohol; A-CSA, petrolatum base with cetearyl alcohol; B-SA, mineral oil/petrolatum base with stearyl alcohol; B-CSA, mineral oil/petrolatum base with cetearyl alcohol. From Ref. 1.

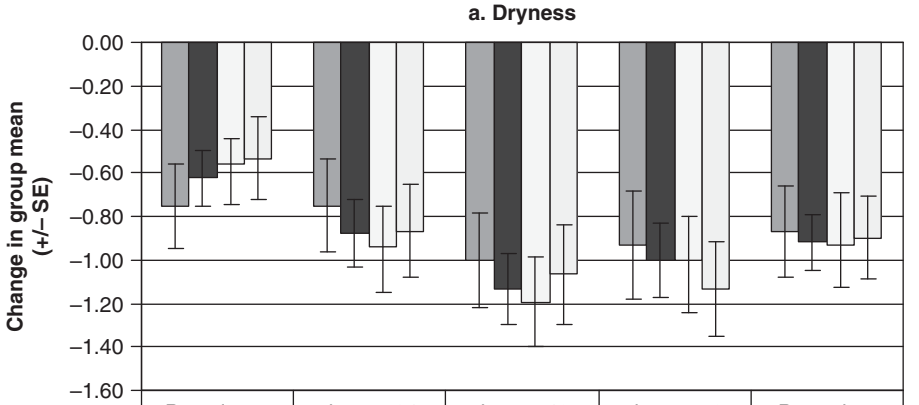
dramatically reduce the improvement in dryness scores. There were differences in the study protocols that may have contributed to this finding. In the study shown in Figure 51.4, a greater number of total tissue wipes was used (200 *vs* 130), and a higher concentration of SLS was used in the pretreatment (0.25% *vs* 0.1%) compared with the study shown in Figure 51.5. This may account for some differences in the results. However, an evaluation of experiments in which the identical tissue and lotion combination was used, along with the two different concentrations of SLS, indicates that the scores resulting from pretreating with 0.25% SLS are similar in severity and the time of recovery to the scores resulting from pretreating with 0.1% SLS (data not shown). Therefore, it is unlikely that the difference in the concentration of SLS used in pretreatment accounts for the different results in these two experiments.

Interestingly, the increase in erythema scores normally seen in all other studies was not observed in the results shown in Figure 51.5. After essentially no increase immediately after the tissue wipe, erythema dropped at each subsequent scoring time point. In all other studies, erythema tended to increase to a greater extent immediately after the tissue wipe, sometimes staying elevated for 24 or 48 hours. It is not obvious why this should be the only study in which erythema scores decreased so consistently.

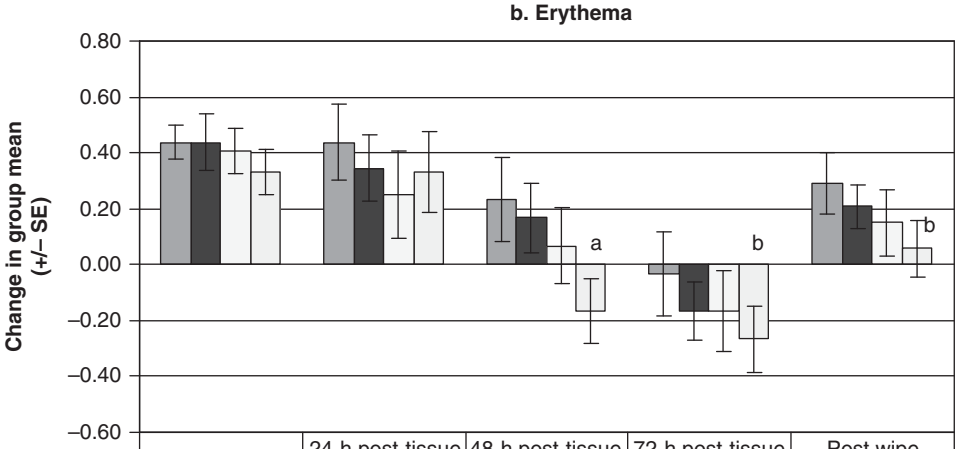
The relative effects of cetearyl and stearyl alcohol were compared in two different base formulations, as shown in Figure 51.6. These two alcohols had very similar effects in the two lotion formulations (lotion A which is a petrolatum base and lotion B which is a mineral oil/petrolatum base). The cetearyl alcohol showed slightly greater reductions in dryness than the stearyl alcohol in the mineral oil/petrolatum formula (lotion B) *versus* the petrolatum formula (lotion A) (Fig. 51.6A, B). Both alcohols produced similar levels of erythema (Fig. 51.6C, D).

Figure 51.7 shows the comparison of four formulations that differ in the base ingredients. Lotion P-CSA has a mineral oil base. Lotion B-CSA has a mixture of mineral oil and petrolatum. Lotion A-CSA has a petrolatum base. Lotion C is a proprietary mixture of fatty acid sucrose esters. All four formulations had comparable benefits with regard to dryness (Fig. 51.7A). However, the test sites treated with proprietary formulation (C) exhibited significantly less erythema (Fig. 51.7B) than the other formulations at the final two scoring time points (48 and 72 hours post-tissue wipe), and significantly less overall (postbaseline average) indicating that this lotion formula base may provide greater skin benefits than the other mineral oil or petrolatum formulations.

The potential skin benefits from lotion-coated facial tissues are the result of a number of factors, including the condition of the skin, the type of tissue substrate, the composition of the lotion, the amount of lotion on the tissue and the kinetics of the lotion transfer to the skin. Developing the best tissue product requires balancing the interactions of all these factors to find a combination of lotion and substrate that maximizes the skin benefits, while minimizing the potential for the chemical and mechanical irritation of damaged skin. Testing on normal skin will not adequately account for all of these factors. However, when the stratum corneum and its barrier properties were slightly damaged by pretreatment with SLS, materials from the lotion formulation can



	Post-tissue wipe	-h post-24 tissue wipe	-h post-48 tissue wipe	-h post-72 tissue wipe	Post wipe average
P-CSA	0.75–	0.75–	1.00–	0.93–	0.87–
B-CSA	0.63–	0.88–	1.13–	1.00–	0.92–
A-CSA	0.56–	0.94–	1.20–	1.00–	0.93–
C	0.53–	0.87–	1.07–	1.13–	0.90–



	Post-tissue wipe	24-h post-tissue wipe	48-h post-tissue wipe	72-h post-tissue wipe	Post wipe average
P-CSA	0.44	0.44	0.23	–0.03	0.29
B-CSA	0.44	0.34	0.17	–0.17	0.21
A-CSA	0.41	0.25	0.07	–0.17	0.15
C	0.33	0.33	–0.17	–0.27	0.06

FIGURE 51.7 Comparison of emollient bases. The test conditions were identical to those described for Figure 51.6. Comparisons were performed using analysis of variance. [a]C different from P-CSA, B-CSA, and A-CSA ($P < 0.05$). [b]C different from P-CSA ($P < 0.05$). *Abbreviations*: P-CSA, mineral oil base with cetearyl alcohol; B-CSA, mineral oil/petrolatum base with cetearyl alcohol; A-CSA, petrolatum base with cetearyl alcohol; C, fatty acid sucrose esters. From Ref. 1.

penetrate more easily, leading to an increase in chemical irritation and an ability to differentiate between products. This modification of the FCAT can be used to compare various lotion formulations for skin benefits and healing properties, and to qualitatively rank the benefits of various formulation options.

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52 Evaluating mechanical and chemical irritation using the behind-the-knee test: A review

Miranda A. Farage

INTRODUCTION

The evaluation of the potential irritant effects to skin is an important part of the overall safety assessment for many consumer products. Such an evaluation usually includes some form of in-use clinical or simulated use testing, with third party evaluation of the skin condition by a trained grader. The nature of the in-use testing that is conducted is often dictated by the product being tested. For example, laundry products are traditionally tested in protocols requiring immersion in solutions of the product, or wear tests of laundered fabrics (1,2). Personal cleansing products and baby wipes are tested using forearm wash or wipe tests (3–6). Catamenial (feminine protection) products are typically tested in in-use clinical studies in which volunteer panelists use the product in place of their normal product (7).

Unfortunately, although the data generated in the in-use clinical studies have been valuable in completing safety assessments, the planning and conduct of such studies presents difficulties. For example, in clinical studies on catamenial products, large panel sizes of at least 30 women per test group must be used to detect the differences in skin effects. Because the tests are often designed so that start dates coincide with the panelists' menstrual cycles, results may not be available for a minimum of 4–5 weeks from study initiation. Grading is done by visual assessment of the genitalia and is, therefore, intrusive for the panelists. Each panelist can test only one product at any one time, making side-by-side comparisons more difficult. The in-use test results can be confounded by changing conditions in the vulvar and vaginal regions due to microbiologic and/or hormonal differences throughout the menstrual cycle. Panelists may have a broad range of pad wearing and hygiene habits. Finally, investigations into some areas, such as testing on compromised skin, are not possible due to the nature of the in-use test.

The high cost, slow turnaround time, and possible confounding factors associated with in-use clinical testing for catamenial products results in slow and expensive skin safety programs on these materials. In addition, a reliance on clinical testing presents a barrier to the rapid development of new products.

Standard patch testing has been used as an alternative to in-use clinical testing in the past for early evaluations of skin safety during the product development process. However, patch testing evaluates only the inherent chemical irritation caused by a material and is, therefore, incomplete. Patch testing does not evaluate the potential mechanical irritation component, that is, the potential irritation caused by friction that is so important for some product categories. Evaluating mechanical irritation for catamenial products is particularly important since vulvar skin has been demonstrated to have a higher coefficient of friction than other body sites (8).

We developed an alternative test method for evaluating skin effects that would eliminate some of the difficulties of the in-use clinical test system while still providing reliable results on the potential irritant effects. The resulting test system is the "Behind-the-Knee" test method (or BTK test), using the popliteal fossa as a test site. In this test protocol, samples are applied to the back of the knee using an elastic knee band. As the panelists go about their everyday activities, normal movements generate friction between the test sample and the skin at the test site, thereby adding the element of mechanical irritation. Thus, the BTK test protocol evaluates the inherent chemical irritation potential and the potential for mechanical irritation (9–13).

Here, examples of results from BTK tests conducted on a variety of materials are presented. Where possible, direct comparisons have been made to the results of in-use clinical testing conducted on the same materials. The results demonstrate that the BTK test is reproducible, giving consistent and reliable results when the same materials are tested repeatedly. The test is capable of detecting subtle differences between very similar products that are consistent with clinical testing and other evaluations.

This is a versatile test system capable of providing meaningful results on a variety of different types of materials. In addition, the BTK test provides results consistent with the results of in-use clinical testing conducted on the same materials, and additional data from over 20 years of in-use clinical tests (14). Unlike the in-use clinical test method, two products can be tested on the same panelist at the same point in time. In fact, by using a common control material and concurrent panels, multiple products can be compared. Investigative programs are possible since the BTK test is easier to conduct for both the investigators and the panelists, providing results in a shorter period of time at a greatly reduced cost.

Although the BTK test was developed using catamenial products, the test system provides a valuable alternative for evaluating the skin effects of any material where mechanical irritation may play a role in overall skin irritation and consumer satisfaction. It has potential applicability for evaluating textiles, facial tissues, baby and adult diapers, and laundry products that may leave residues on fabrics.

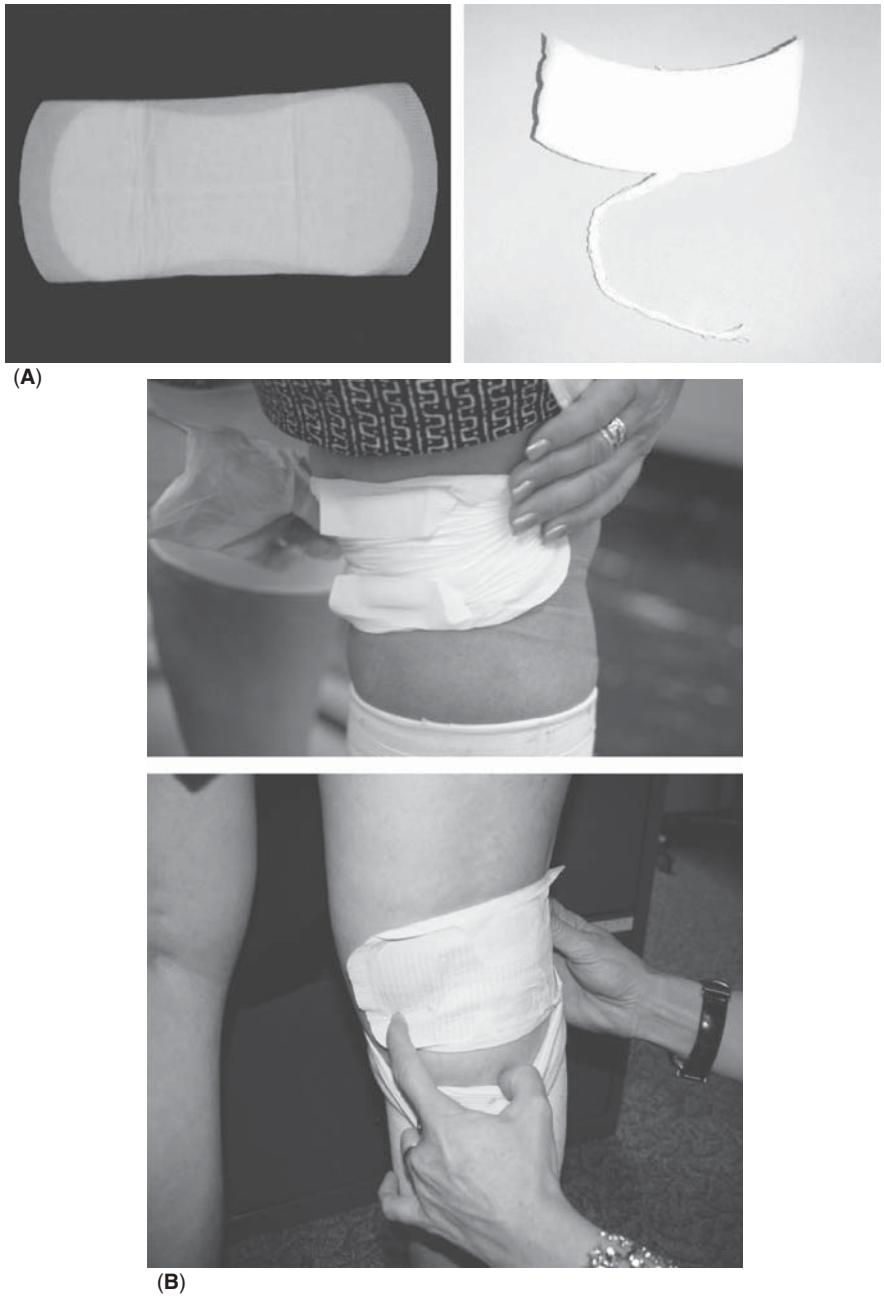

FIGURE 52.1 Test sample application in the "behind-the-knee" test. Testing can be done on a variety of materials, including menstrual pads and uncompressed tampons (**A**). Test materials are placed horizontally on the popliteal fossa, and held in place by an elastic knee band of the appropriate size (**B**).

METHODOLOGY

Basic BTK Test Protocol

The BTK test protocols have been described in previous publications (9–13), and in the American Society for Testing Materials (ASTM) standard protocol (15). Test material was placed horizontally and held in place on the popliteal fossa by an elastic knee band of appropriate size, as shown in Figure 52.1. Menstrual pads, pantiliners, topsheets and fabrics were tested as is. Tampons are marketed in a highly compressed, cylindrical configuration that would make poor contact with the skin in the BTK test. Therefore, these products were tested as uncompressed samples.

Test materials were removed by the panelists 30–60 minutes prior to returning to the laboratory for grading and/or reapplication of test materials. Exposures varied from 24 hours per day for 3–5 consecutive days in early experiments to 6 hours per day for 4 or 5 consecutive days in more recent experiments.

Visual grading of the BTK test sites was conducted by an expert grader under a 100 W incandescent daylight blue bulb. Scoring was done using a previously described scale of "0" to "4", where "0" is no apparent cutaneous involvement and "4" is moderate-to-severe, spreading erythema and/or edema (9,15). The same grader was used throughout an experiment, and the grader was not aware of the treatment assignments.

In the BTK test protocol, the integrity of the skin can be compromised by tape stripping using Blenderm® tape (3M, St Paul, Minnesota, U.S.) prior to the first application. In some experiments, this was done by repeatedly applying tape to the area up to 20 times, or until the skin exhibited an erythema score of 1.0–1.5 as per the grading scale described in the previous paragraph.

Study participants were healthy adult volunteers who had signed an informed consent with no medical or skin condition likely to interfere with the test. Unlike the clinical studies, both male and female panelists can be recruited for the BTK studies. Participants filled out a brief questionnaire each day to record any discomfort (itching, chafing, burning, and so forth).

Materials Tested

Test materials included fabric, menstrual pads and pantiliners, topsheets from pads and pantiliners, products with and without lotion coatings, tampons, and interlabial pads. A summary of the materials tested and the corresponding sample codes are provided in Table 52.1.

Standard Patch Test and Clinical Methodology

Results of the BTK test were compared with those from standard patch testing or clinical testing protocols. Standard patch testing was conducted as previously described (11). Briefly, patch sites for test materials and conditions were randomized, and test samples were applied via an occlusive patch. Sites were marked with 0.5% gentian violet to aid in visual grading and to ensure that the patches were applied to identical sites each day for the duration of the test. Test materials were removed by the panelists 24 hours after application, and subjects returned to the laboratory for grading and/or reapplication of test materials 30–60 minutes later. Test sites were scored in a manner identical to the popliteal fossa test sites, as described in Section "Basic BTK Test Protocol."

Study designs for the in-use clinicals are provided in Table 52.2. In the clinical studies on menstrual pads (samples AGT, M, NL, and N), panelists were randomly assigned one of the two test products. They were provided with a product and asked to use it during one or two menstrual periods in place of their normal product. For the study on the interlabial pads, panelists in the test group were asked to use the test product (sample IL2) for a minimum period of 8 hours daily for an entire menstrual cycle (approximately 1 month). A control menstrual pad (A) was to be worn in addition to the test product during menses. The control group used a currently marketed pantiliner (sample P) in place of the interlabial test product. The tampon studies (samples T, U, S, and R) were a crossover design. Half the panelists used one tampon for one menstrual period, then switched to the other for their next period. The other half of the panelists used the tampons in reverse order.

In the in-use clinical studies on menstrual pads, skin condition was assessed by visual grading of the external genitalia for evidence of irritation based on the following scale: 0 = normal skin, 1 = slight erythema, 2 = moderate erythema, 3 = severe erythema, 4 = edema/induration, 5 = skin fissuring, 6 = spreading reaction, and 7 = vesicles/bullae. This grading scale has been previously described in detail (7). Seven sites were graded separately: mon pubis, labia majora, labia minora, introitus, vestibule, perineal body, and upper thighs. In some studies the clitoris and buttocks were also evaluated.

In the in-use clinicals on tampons, erythema was evaluated via colposcopic examination based on the following scale: 0 = none, 1 = faint, 2 = moderate, 3 = moderate-to-severe, and 4 = severe.

TABLE 52.1
Summary of Materials Tested in BTK Test

Type of Sample	Number of Times Tested	Test Product Sample Codes
Fabrics	9	Burlap, Satin
Menstrual pads	27	A, B, C, E, H, N, M, AGT
Ultrathin pads	12	AU, BU, TU
Topsheets	12	AT, BT, GT
Lotion-coated samples	7	NL, AUL, ATL, BTL
Tampons	4	T, U, R, S
Pantiliners	1	P
Interlabial pad	1	IL2, IL25

Abbreviation: BTK, behind-the-knee test.

TABLE 52.2
Summary of Test Designs for In-Use Clinical Studies

Test Samples					
Code	Description	Code	Description	Study Design	Evaluation Period
AGT:	Menstrual pad with hydrofilm-type polyethylene topsheet	M:	Menstrual pad with non-woven topsheet	Side-by-side comparison	Panelists used same test product used for two menstrual cycles
NL:	Test menstrual pad with lotion and perfume	N:	Test menstrual pad without perfume	Side-by-side comparison	Panelists used one test product for one menstrual cycle
IL2:	Interlabial pad composed of cotton/rayon core with rayon topsheet	P:	Non-winged, regular length, unscented pantiliner composed of pulp absorbent core with polyethylene	Side-by-side comparison	Panelists used one test product for one menstrual cycle
T:	Super plus absorbency tampon (absorbs 12–15 g) composed of rayon with a polyethylene/polypropylene bi-component outer layer	U:	Ultra absorbency tampon (absorbs 15–18 g) composed of rayon with a polyethylene/polypropylene bicomponent outer layer	Crossover design	Panelists used one test product for one menstrual cycle, then switched to other test product for a second cycle
S:	Tampon composed of cotton and rayon absorbent fiber with rayon/polyester outer layer	R:	Tampon composed of cotton and rayon absorbent fiber with rayon outer layer	Crossover design	Panelists used one test product for one menstrual cycle, then switched to other test product for a second cycle

Six sites are graded separately: labia minora, introitus, lower and middle vaginal walls, upper vagina, and cervix.

Analyses of Data

In the BTK studies, paired comparisons were conducted using a Wilcoxon signed-rank test on the irritation scores collected after completion of all test sample applications, unless otherwise stated in the legends of the appropriate tables (16). In the in-use clinical studies, if the data were normally distributed, evaluation was based on a paired *t* test. If data were not normally distributed, evaluation was based on a signed-rank sum test (17–19).

RESULTS

Reproducibility and Versatility of the BTK Test

The method has been used in repeated studies on numerous materials, and comparisons between the same materials provide consistent results using a variety of exposure regimens and other protocol variations. Table 52.3 summarizes the results of multiple experiments comparing two control materials for mechanical irritation (burlap and satin) using different durations of exposure. Regardless of the specific exposure regimen, that is, 6 hours per day for 4 days, or 24 hours per day for 3 or 4 days, the burlap produced a significantly higher level of irritation than satin, based on the mean erythema scores.

Repeated studies were also conducted on two menstrual pads with different topsheets: pads A and B. These products have been used repeatedly as controls and standards in the in-use clinical studies, and in other studies for irritation such as the standard patch tests. Both products are mild to skin in both our clinical and patch testing, and during normal use by the panelists. However, the panelists consistently preferred pad B based on a more pleasing feel and texture (data not shown). Table 52.3 summarizes comparisons of these two samples using 6-hour exposures under four exposure conditions that simulate some of the physiologic conditions that may occur during normal product use. The exposure conditions included the standard protocol (i.e., dry samples on intact skin), and three protocol variations, including dry samples on compromised (tape-stripped) skin, wet samples on intact skin, and wet samples on tape-stripped skin. In all the cases, pad A yielded higher mean scores for erythema than pad B.

Table 52.4 shows the results on materials and products where alternative data enabled us to predict the likely results. In the top four examples (Table 52.4A), in-use clinical studies were conducted on the identical materials. In these studies, there were no significant differences in the level of irritation between the products. BTK tests on the same materials yielded the same results as the in-use clinical tests. In the remaining examples (Table 52.4B), the likely results could be predicted based on close similarities between the test materials and/or previous consumer evaluations, as indicated in the table. In all the cases, the BTK test yielded the expected result.

Ability to Test both Mechanical and Chemical Irritation

In some studies, standard patch tests were applied to the same panelists participating in the BTK test using identical protocol modifications, that is, wet samples and/or skin compromised by tape stripping. We compared the level of irritation in the BTK test to that shown in the standard patch test to develop a qualitative understanding around the proportion of the overall irritation that could be attributed to mechanical irritation *versus* chemical irritation. In the examples shown in Figure 52.2, the mean erythema score for the standard patch test is represented as a percentage of the mean score for the BTK. With one exception, the standard patch test produced overall mean irritation results that were 65–85% of the BTK method. The higher overall mean scores produced by the BTK method likely represent the mechanical irritation component of the reaction.

Ability to Compare Several Products Tested Concurrently

In some circumstances, it is desirable to compare the potential irritant effects of more than two products. Table 52.5 shows the result of an experiment in which three panels were run concurrently enabling comparison of the effects of four products. In this study an experimental topsheet material (topsheet GT) was compared with three menstrual pads (M, E and H) using three concurrent panels. The topsheet material was similar in irritation potential to all three pads. Since the three panels were run concurrently, and one sample was common to all three panels (i.e., topsheet GT), it was possible to statistically compare the three pads to each other. As shown in Table 52.5, when this statistical

TABLE 52.3

Similar Results with Different Exposure Regimens and Protocol Variations in the BTK Protocol

Exposure Regimen	No. of Panelists	Protocol Variation(s)	Mean Erythema Score ± SE		Significance (*P* value)
			Burlap Fabric	Satin Fabric	
6 hr/day for 4 days	12	None	1.5 ± 0.17	0.55 ± 0.08	<0.002
6 hr/day for 4 days	9	None	1.8 ± 0.12	0.38 ± 0.12	<0.01
24 hr/day for 3 days	10	None	1.7 ± 0.15	0.60 ± 0.22	<0.01
24 hr/day for 4 days	13	None	1.9 ± 0.08	0.77 ± 0.26	<0.01
			Menstrual pad A	Menstrual pad B	
6 hr/day for 4 days	17	None	1.7 ± 0.06	1.1 ± 0.10	<0.002
6 hr/day for 5 days	16	Compromised skin	2.0 ± 0.14	1.4 ± 0.11	<0.05
6 hr/day for 5 days	18	Wet sample	1.6 ± 0.10	1.3 ± 0.09	<0.05
6 hr/day for 5 days	17	Compromised skin and wet sample	1.8 ± 0.11	1.3 ± 0.11	<0.01

Note: Mean scores for erythema (± SE) at completion of the study were determined. Comparison of mean scores was done using Wilcoxon signed-rank test.

TABLE 52.4

BTK Test Results on Diverse Product and Material Types

A. Comparison of BTK Test Results with In-Use Clinical Results

	In-Use Clinical					BTK Test			
Materials Tested	**No. of Panelists**	**Results (Mean Erythema ± SE)**			**Significance**	**No. of Panelists**	**Results (Mean Erythema ± SE)**		**Significance**
Pads AGT *vs* M	55		Labia majora	Labia minora	NS	42	AGT:	0.96 ± 0.13	NS
	60	AGT:	0.22 ± 0.04	0.01 ± 0.01			M:	1.15 ± 0.13	
		M:	0.20 ± 0.03	0.0 ± 0.0					
Pad N *vs*	59		Labia majora	Labia minora	NS	41	N:	2.0 ± 0.07	NS
lotioned pad	60	N:	0.22 ± 0.05	0.06 ± 0.03			NL:	2.0 ± 0.05 [a]	
NL		NL:	0.10 ± 0.04	0.02 ± 0.02					
Interlabial pad	29		Labia majora	Labia minora	NS	22	IL2:	1.4 ± 0.09	NS
IL2 *vs*	36	IL2:	0.97 ± 0.11	1.24 ± 0.13			P:	1.4 ± 0.11	
pantiliner P		P:	0.83 ± 0.12	1.14 ± 0.12					
Tampons			Lower vaginal wall	Middle vaginal	NS	17	T:	1.3 ± 0.09	NS
T *vs* U	43	T:	0.20 ± 0.04	wall			U:	1.2 ± 0.08	
		U:	0.08 ± 0.03	0.17 ± 0.04					
				0.10 ± 0.03					
Tampons	67		Lower vaginal wall	Middle vaginal	NS	15	R:	0.9 ± 0.09	NS
R *vs* S		R:	0.52 ± 0.04	wall			S:	1.0 ± 0.10	
		S:	0.49 ± 0.04	0.38 ± 0.04					
				0.45 ± 0.04					

B. Comparison of BTK Test Results with Expected Results

			BTK test result			
Materials Tested	**Expected Result**	**No. of Panelists**	**Results (Mean Erythema ± SE)**		**Significance (*P* value)**	**Conclusion**
Pads	In previous consumer	17	A:	1.7 ± 0.06	<0.05	Pad A gave a significantly
A *vs* B	evaluations, pad B was preferred over pad A		B:	1.1 ± 0.09		higher mean score than pad B
Topsheets	In previous consumer	11	AT:	1.3 ± 0.25	<0.05	Topsheet AT gave a signifi-
AT *vs* BT	evaluations, topsheet BT was preferred over topsheet AT		BT:	0.59 ± 0.16		cantly higher mean score than topsheet BT
Fabrics: Burlap *vs*	Based on the characteristics	12	Burlap:	1.5 ± 0.17	<0.002	Burlap gave a significantly
Satin	of the fabrics, burlap was expected to give a significantly higher score than satin		Satin:	0.6 ± 0.08		higher mean score than satin

Note: In-use clinical and BTK studies were conducted as described in the Methodology section (Standard Patch Test, and Clinical Methodology and Basic BTK Test Protocol subsections, respectively).

[a]Each 6-hr exposure consisted of fresh lotioned samples applied at time 0 and at 3 hr.

Abbreviations: BTK, "behind-the-knee" test; NS, not significant.

comparison was done, menstrual pads M, E, and H were similar in irritation potential.

DISCUSSION

An array of materials can be tested for a combination of mechanical and chemical irritation properties in the BTK test (Table 52.1). We have successfully tested menstrual pads, topsheets, tampons, and fabric. The ability to test fabric samples indicates that the test protocol may have applicability for evaluating the properties of fabric materials themselves, or for products and chemicals that may deposit on fabrics, such as fabric softeners and detergents.

The results presented in Table 52.3 illustrate the reproducibility of the BTK test. Comparisons between the same two materials in repeated studies using different exposure regimens and/or protocol variations produced consistent results. In early development studies satin and burlap were used as control materials. It was expected that these two materials would differ markedly in the ability to cause mechanical irritation from friction. As expected, burlap produced a significantly higher level of irritation than satin, whether the exposure was for 6 hours per day or 24 hours per day. We have

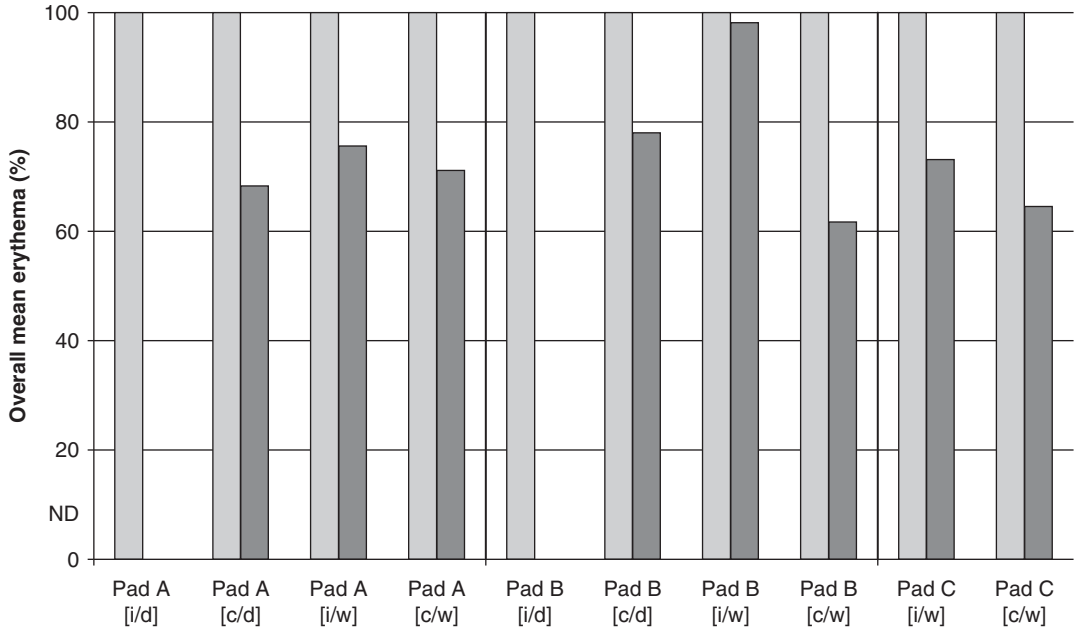

FIGURE 52.2 Representation of the relative contribution of mechanical and chemical irritation. For menstrual pads A, B, and C, the overall mean erythema scores from the standard patch test are expressed as a percentage of the overall mean erythema scores from the BTK test. The data from the popliteal fossa and upper arm test sites were generated in parallel using the same panelists. These studies have been described in detail in earlier publications (9–13). *Abbreviations*: BKT, "behind-the-knee" test; ND, not done; i/d, intact skin, dry sample; c/d, tape stripped, compromised skin, dry sample; i/w, intact skin, wet sample; c/w, tape stripped, compromised skin, wet sample; BTK test result (mechanical and chemical irritation); and patch test (chemical irritation only).

TABLE 52.5
Using a Common Material as a Basis to Compare Multiple Samples

		Number of Panelists	Material Tested	Mean ± SE	Material Tested	Mean ± SE	Significance
Intratrial comparison	Group a	13	Topsheet GT	0.96 ± 0.13	Pad M	1.2 ± 0.13	NS
	Group b	13	Topsheet GT	1.1 ± 0.17	Pad E	1.1 ± 0.16	NS
	Group c	14	Topsheet GT	1.0 ± 0.12	Pad H	0.96 ± 0.08	NS
Intertrial comparison	Groups a & b	13 & 13	Pad M	1.2 ± 0.13	Pad E	1.1 ± 0.16	NS
	Groups b & c	13 & 14	Pad E	1.1 ± 0.16	Pad H	0.96 ± 0.08	NS
	Groups c & a	14 & 13	Pad H	0.96 ± 0.08	Pad M	1.2 ± 0.13	NS

Note: In this study panelists wore the test materials for 6 hr/day for 5 days. An experimental topsheet material (topsheet GT) was compared with three menstrual pads (M, E, and H) using three separate groups of panelists tested concurrently (groups a, b, and c shown as "intratrial comparison"). Since the topsheet material was a common leg in all three separate groups, the other three samples (pads M, E, and H) could be compared statistically for differences in irritation reactions (intertrial comparison). Comparison of the final day scores are given. The intratrial comparisons were done using analysis of variance or analysis of co-variance. The intertrial comparison was done using Cochran–Mantel–Haentzel.
Abbreviation: NS, not significant.

also obtained consistent results in repeated tests using other materials and different exposure regimens (data not shown). Comments from participating panelists indicated that the longer exposure time of 24 hours became uncomfortable for some panelists, with no real benefit to the quality of the results. Therefore, the current standard protocol calls for 6-hour exposure times (15). However, longer exposures can be used successfully in investigative studies.

Table 52.3 also demonstrates the ability of the test to detect subtle differences between two very similar products; menstrual pads (A and B). Both products are mild to skin and considered nonirritating. However, the BTK test consistently demonstrated that pad A yielded higher mean erythema scores than pad B, regardless of the specific test conditions. This is consistent with results of consumer and panelist evaluations that indicate panelists prefer pad B over pad A because of a more pleasing feel and texture (data not shown).

The preference for pad B based on the sensations caused by the products (i.e., sensory effects) has been studied in the course of BTK testing (10). In some studies, panelists kept a daily diary of skin problems experienced at the test sites. Panelists were asked if they experienced specific sensations, such as the sample rubbing against the skin or sticking to the skin, chafing, burning, itching, pain, or any other discomfort. At several scoring time points, the percentage of subjects complaining of the sensations of burning, sticking, or pain was significantly lower for standard pad B when compared with standard pad A.

Table 52.3 also illustrates the ability to incorporate different test conditions in the BTK test. In addition to testing products as is, we tested samples under varying physiologic conditions known to occur during use of catamenial products, including conditions where the test materials were wet, and/or the skin may have been compromised. Even under conditions that represent the

most extreme physiologic conditions of product use, that is, wet product on compromised skin, the BTK test produces reliable results. Such investigations are not always possible in in-use clinical studies, where the specific test site and ethical considerations may preclude any steps to compromise the skin.

The ability to evaluate potential irritation using a variety of conditions under highly controlled circumstances has implications for products other than catamenial products. For example, with baby and adult diapers, the precise conditions encountered may be difficult to control in an in-use clinical. Different use habits and patterns would influence the degree of moisture in the product. Confounding factors, such as pre-existing rashes or irritation, may exist and compromise the results. However, in the BTK, single variables can be controlled and evaluated within a short time, providing more precise results and increasing the ability to pinpoint any contributions of the product to the overall skin effects.

We have tested over 25 different materials in over 35 BTK studies, and the test method has proved reliable and versatile in testing a wide variety of materials, including fabric, menstrual pads, topsheets, interlabial pads, pantiliners, tampons, and lotion coatings on products. Table 52.4 illustrates the utility of the BTK test method for testing various product types. It is possible to test products of different shapes and end uses, for example, externally worn catamenial pads, interlabial pads, and tampons, with no alterations to the basic protocol. For some materials, in-use clinical studies were available on the identical test products compared in the BTK test. These are given in Table 52.4A. In all cases, the BTK test gave similar results to the in-use study. For other materials, alternative data were available that enabled a prediction as to the expected result in the BTK test (Table 52.4B). This alternative data included a knowledge of the nature of the materials being tested (as in the case of the fabrics burlap and satin), or evaluations and previous testing (as in the case of menstrual pads A and B, and topheets AT and BT). In all cases, the BTK test gave the expected result. It is noteworthy that several of the BTK studies shown in Table 52.4 have been repeated several times, giving results consistent with those shown in the table.

A main use for in-use clinical testing is to establish the safety of a new product or product improvement by demonstrating that the test sample does not result in an increase in adverse skin effects compared with the control sample. For the products presented in Table 52.4A, clinical testing was conducted to demonstrate no change in the irritation potential for the product safety assessment, claims support, and for the purposes of regulatory filings. Given the nature of the in-use clinical test, it would not be ethical to conduct studies with materials that would be expected to produce frank irritation. Furthermore, given the expense and logistic difficulties associated with the in-use clinical studies, the number of studies that can be conducted for purely investigative purposes is limited. However, we were able to select products that had been tested recently in the in-use clinical, and test these same products in the BTK test, thus generating side-by-side comparisons to complete the validation of the BTK test. In all cases, the BTK test duplicated the results observed in the in-use clinical study conducted on the same materials in terms of similarities and differences in irritation, based on the data presented.

The potential irritation that may be caused by catemenial pads is a combination of the inherent irritation potential of the chemical components of the products, and mechanical irritation that occurs as a result of wearing the products in close proximity to the skin for prolonged periods of time during normal movement and activity. The standard patch test method does not evaluate mechanical irritation, and therefore, provides an incomplete picture. Figure 52.2 illustrates the contribution of mechanical irritation to the overall irritation potential. As shown in this figure, when the same products are tested on the same panelists under the same experimental conditions, the BTK test site almost invariably exhibits more irritation than the standard patch test site. This difference is due to the mechanical irritation caused by the friction of the test material against the skin.

The BTK test can also be used to compare two or more products, as shown in Table 52.5. Separate panels can be conducted concurrently, sharing a single common test material. The common test material acts as a control, allowing the statistical comparison of the test materials applied to the other test site. Such side-by-side product comparisons are not possible using in-use testing.

Investigative programs are difficult to conduct using in-use clinical testing. However, with the BTK test, we have conducted many investigative studies to aid product development efforts. One such program involved evaluating subjective sensations of irritant effects, such as feelings of itching, burning, sticking, and so on, and correlating these so-called sensory effects with outward evidence of irritation. In some studies, we found an association between the sensory effects reported by panelists and the degree of irritation determined by scoring for erythema (10). Additional details of this program are provided in a separate publication (20).

An additional example of an investigative program is quantitative lotion transfer assessments. In these experiments, we studied the transfer of lotion from feminine protection products to the skin, and compared the results to those from in-use clinical studies (21,22). Both the BTK and the clinical protocols provided a means of evaluating the transfer of lotion formulations from feminine protection pads. However, the clinical protocol was subject to variables that did not impact the BTK protocol, such as differences in wear times, contact pressure, activity levels, and so on. Therefore, the BTK test method yielded more consistent results. Furthermore, the BTK protocol enabled side-by-side comparisons of two products. The in-use clinical study necessitated sequential exposure. While both protocols demonstrated that the amount of lotion transferred from the product to the skin was proportional to the starting amount of lotion on the product, only the BTK showed a difference in the amount transferred depending on the type of absorbent core (superabsorbent gelling material or cellulose).

The BTK test is the result of a program to develop a method for evaluating skin effects for products used in the urogenital region that eliminates the difficulties of the in-use clinical test without compromising the quality of the results. The specific advantages of the BTK test are summarized in Table 52.6. Both testing approaches provide reproducible and reliable results, and can discriminate between very similar products. However, the flexibility, ease of implementation, rapid turnaround time, and lower cost of BTK test make it a much more useful tool for safety testing, investigative programs, product development efforts, and claims support. This BTK test system provides a potentially valuable alternative for evaluating the skin effects of any material where mechanical (in addition to chemical) irritation may play a role in overall skin irritation and consumer satisfaction, with potential applicability for textiles, facial tissues, baby and adult diapers, and laundry products that may leave residues on fabrics. Furthermore, the BTK clinical test is now a global ASTM standard protocol.

TABLE 52.6
Comparison of Characteristics of the In-Use Clinical and Popliteal Fossa Test Systems

Characteristics	In-Use Clinical Test System	BTK System
Quality of results	Provides reproducible, reliable results.	Provides reproducible, reliable results.
	Capable of detecting subtle differences between very similar products.	Capable of detecting subtle differences between very similar products.
	Side-by-side product comparisons not possible. Each panelist can test only one product at a time.	Conducive to side-by-side product comparisons. Each panelist can test two products concurrently.
Ease of implementation	Cumbersome.	Simple.
	Staggered start necessary. Start date for each panelist must coincide with the menstrual cycle.	Start date is independent of the menstrual cycle.
	Panel size of at least 30 per product.	Panel size of 15–20 for two products.
	Panel must be composed of the specific target consumer type, that is, menstruating women for catamenial products, incontinent adults for adult diapers, etc.	Many products may be tested on healthy, male and female adult volunteers.
	Grading is intrusive for panelists.	Grading is not intrusive (similar to the standard patch test).
Confounding factors	Panelists may have a broad range of pad wearing and hygiene habits that may impact results.	Wearing time and test conditions can be controlled.
	Results can be confounded by changes in microbial distribution during the menstrual cycle.	Results independent of changes in microbial distribution.
Turnaround time	Results available in a minimum of 4–5 weeks from study initiation due to staggered start. Start date for each panelist must coincide with the menstrual cycle.	Results are available 1–2 weeks after initiation.
Cost	Costly (≥$150,000–$200,000 per study).	Inexpensive (~$5000 per study).
Usefulness in investigative programs	Investigative programs are not practical due to high cost and slow turnaround time.	Investigative programs are inexpensive and quick.
	Investigative studies, such as testing on compromised skin, cannot be easily incorporated into the protocol.	Investigative studies are easily incorporated into the protocol.

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53 Tests for sensitive skin

Annahita Sarcon, Raja K. Sivamani, Hongbo Zhai, Alessandra Pelosi, Enzo Berardesca, and Howard I. Maibach

INTRODUCTION

Sensitive skin is a condition of subjective cutaneous hyperreactivity to environmental factors or topically applied products. The skin of subjects experiencing this condition reacts more easily to cosmetics, soaps, and sunscreens and often enhance worsening after exposure to dry and cold climate.

Sensitive skin and subjective irritation are widespread because of the use of cosmetics is increasing in countries having economic advantage. The self-declared sensitive skin, is often described on the face, but hands and the scalp can also be involved (1,2). The frequent use of preservatives, perfumes, emulsifiers, and plant extracts in fact enhance risk of adverse local reactions.

The signs of discomfort, such as itching, burning, stinging, and a tight sensation, are commonly present, either associated or not with erythema and scaling. Uncomfortable sensations with no visible signs account for 50% of reported adverse reactions to cosmetics and toiletries (3).

Generally, substances that are not commonly considered irritants are involved in this abnormal response. They include many ingredients of cosmetics, such as dimethyl sulfoxide, benzoyl peroxide preparations, salycilic acid, propylene glycol, amyldimethylaminobenzoic acid, and 2-ethoxyethyl methoxycinnamate (4). The unpleasant sensations appear to be associated with the stimulation of cutaneous nerve endings specialized in pain transmission, called nociceptors.

Some authors (5) hypothesized a correlation between sensitive skin and constitutional anomalies or other triggering factors, such as occupational skin diseases or chronic exposure to irritants; and some others (6) supported that no constitutional factors play a role in the pathogenesis of sensitive skin, although the presence of dermatitis demonstrates a general increase in skin reactivity to primary irritants lasting months.

In different epidemiologic surveys, the correlation between sensitive skin with gender, race, skin type, and age has been studied. No gender-related significant differences have been found in the reaction pattern.

Some authors (7–9) documented a higher reactivity to irritants mostly in females, some others noted that male subjects were significantly more reactive than female subjects (10), but some other experimental studies did not confirm these observations (11,12). In a study by Farage (13) a significantly lower proportion of men 50 years or older perceived general sensitivity (52.9%) *versus* women (78.6%), with no significant differences in the ≤30-year, 31- to 39-years, and 40- to 49-years age groups. Men mainly reported rubbing or friction from contact versus women citing visual evidence of skin irritation.

Conflicting data were also reported on skin sensitivity among races. The epidermal barrier, stratum corneum (SC) has similar thickness in Caucasians and Blacks; however, the SC of fair-skinned subjects contains less cell layers than those of Blacks (14). Although blacks seem to be less reactive and Asians more reactive than Caucasians, data rarely reach a statistical significance (15); in a study by Aramaki, significant subjective-sensory differences were found between Asian and Caucasian women but no differences after sodium lauryl sulfate (SLS) testing, concluding that stronger sensations in Asians can reflect a different cultural behavior rather than measurable differences in skin physiology (16).

Nonetheless, skin morbidity differences have been noted in distinct racial groups. For instance, acne, alopecia, and dyschromia are more common among black patients than Caucasians (17,18).

Studying the correlation between skin reactivity and skin type, subjects with skin type I were found to be more prone to develop sensitive skin (19); most common "stingers" were reported to be light-complexioned persons of celtic ancestry who sunburned easily and tanned poorly (20).

Moreover, skin reactivity tends to decrease with age: by testing croton oil, cationic and anionic surfactants, weak acids, and solvents, less severe skin reactions were observed in older subjects (21). Robinson (22), by testing sodium dodecyl sulfate, decanol, octanoic acid, and acetic acid, confirmed this lower reactivity in the older age cluster of subjects.

Aged skin seems to have a reduced inflammatory response either to irritants or to irritation induced by ultraviolet light (23,24). However, skin reactivity of women at the beginning of the menopause is increased, suggesting a role of estrogen deficiency on the observed impairment of skin barrier function (25). Although it is still debatable the beneficial effect of hormone replacement therapy on aging skin of menopausal women has been confirmed (26).

TESTS FOR SENSITIVE SKIN

Clinical Parameters

It is difficult to find accurate parameters for categorizing skin as sensitive or nonsensitive; this condition often lacks visible, physical, or histologically measurable signs. Subjects with subjective irritation tend to have a less hydrated, less supple, more erythematous, and more teleangiectatic skin compared with the normal population. In particular, significant differences were found for erythema and hydration/dryness (27). Tests for sensitive skin are generally based on the report of sensation induced by topically

applied chemicals. Consequently, the use of self-assessment questionnaires is a valuable method to identify "hyperreactors" (9) and a useful tool for irritancy assessment of cosmetics (28).

SENSORY TESTING METHODS

Psychophysical tests based on the report of sensation induced by topically applied chemical probes have been increasingly used to provide definite information on sensitive skin. These methods of sensory testing can be validated by the use of functional magnetic resonance imaging, which represents one of the most developed forms of neuroimaging. This technique measures changes in blood flow and blood oxygenation in the brain, closely related to neural activity manifested as sensory reaction. When nerve cells are active they consume oxygen carried by hemoglobin in red blood cells from capillaries. The local response to this oxygen utilization is an increase in blood flow to regions of increased neural activity, occurring after a delay of approximately 1–5 seconds. This hemodynamic response rises to a peak over 4–5 seconds, before falling back to baseline (and typically undershooting slightly). This leads to local changes in the relative concentration of oxyhemoglobin and deoxyhemoglobin, and changes in local cerebral blood volume in addition to changes in local cerebral blood flow (29).

Quantitation of Cutaneous Thermal Sensation

In dermatology, thermal sensation testing analysis is the most utilized quantitative sensory testing technique (30). It assesses function in free nerve endings and their associated small myelinated and nonmyelinated fibers. This method is able to measure quantitatively the threshold for warm and cold sensation as well as hot and cold pain.

A small device, called thermode, based on Peltier elements, is in contact with the subject's skin. It consists of semiconductor junctions, which produce a temperature gradient between the upper and then lower stimulator surfaces produced by an electric current. In the center of the thermode a thermocouple records the temperature.

Thermal sensory test (TSA 2001; Medoc Company, Ramat Yshai, Israel) is considered one of the most advanced portable thermal sensory testing devices.

Basically, it measures the hot or cold threshold and the suprathreshold pain magnitude (Table 53.1).

TSA operates between 0°C and 54°C. The thermode in contact with the skin produces a stimulus whose intensity increases or decreases until the subject feels the sensation.

TABLE 53.1
Thermal Sensory Test

Parameter Monitored	Sensory Fibers
Warm sensation	C-fiber (1–2°C above adaptation temperature)
Cold sensation	A-delta fibers (1–2°C above adaptation temperature)
Heat-induced pain	Mostly C-fiber (45°C)
Cold-induced pain	Combination of both C- and A-delta fibers (10°C)

As the sensation is felt the subject is asked to press a button. The test is then repeated two more times to get a mean value. Using this method, artifacts can occur due to the lag time needed for the stimulus to reach the brain. This inconvenience can be avoided by using relatively slow rates of increasing stimuli.

The stimulus can also be increased stepwise and the subject is told to say whether or not the sensation is felt. When a positive answer is given, the stimulus is decreased by half the initial step and so on, until no sensation is felt. The subject's response determines the intensity of the next stimulus. The limitation of this second method is that a longer performance time is required.

Stinging Test

Stinging test represents a method for the assessment of skin neurosensitivity. Stinging seems to be a variant of pain that develops rapidly and fades quickly any time the appropriate sensory nerve is stimulated. The test relies on the intensity of stinging sensation induced by chemicals applied on the nasolabial fold (20). Procedure differs depending on the chemical utilized.

Lactic Acid

The lactic acid stinging test was reported to identify persons as "stingers," who generally show an increased tendency to experience subjective irritant reactions to cosmetic products (19,20). After a 5–10 minute facial sauna, an aqueous lactic acid solution (5% or 10% according to different methods), is rubbed with a cotton swab on the test site while an inert control substance, such as saline solution, is applied to the contralateral test site. After application, within a few minutes, a moderate-to-severe stinging sensation occurs for the "stingers group." Subjects are then asked to describe the intensity of the sensation using a point scale. Hyperreactors, particularly those with a positive dermatologic history, have higher scores. Using this screening procedure, 20% of the subjects exposed to 5% lactic acid in a hot, humid environment, were found to develop a stinging response (20). Lammintausta et al. (31) confirmed these observations identifying 18% of their study subjects as stingers. In addition, stingers were found to develop stronger reactions to materials causing nonimmunologic contact urticaria, to have increased transepidermal water loss (TEWL) and blood flow velocimetry values after application of an irritant under patch test.

Capsaicine

An alternative test involves the application of capsaicine. A new procedure assessed by l'Oreal Recherche (32) appears to be more accurate and reliable for the diagnosis of sensitive skin. After a facial cleansing, five increasing capsaicine concentrations in 10% ethanol aqueous solutions are applied on the nasolabial folds. The application of the vehicle alone serves as control and to exclude subjects who feel any discomfort sensation prior to capsaicine application. The formulation of capsaicine in hydroalcoholic solution accelerates the action of capsaicine on the face in comparison with the previously used 0.075% capsaicine emulsion, without being associated with painful sensation.

The capsaicine detection thresholds are more strongly linked to self-declared sensitive skin than the lactic acid stinging test. A recent study in 2009 showed, Asian women that tended to have

higher capsaicin detection thresholds than Caucasians, but lower thresholds than Africans. Nevertheless, the distribution did not greatly differ between the three ethnicities; hence changes in various ethnicities are minimal (32).

Dimethylsulfoxide

The alternative application of 90% aqueous dimethylsulfoxide has not the same efficacy of lactic acid or capsaicine stinging test and, after application, intense burning, tender wheal, and persistent erythema often occur in stingers.

Nicotinate and Sodium Lauryl Sulfate Occlusion Test

A different approach to identify sensitive skin relies on vasodilation of the skin as opposed to cutaneous stinging. Methyl nicotinate, a strong vasodilator, is applied to the upper third of the ventral forearm in concentrations ranging from 1.4% to 13.7% for a 15-second period. The vasodilatory effect is assessed by observing the erythema and the use of laser Doppler velocimetry (LDV). Increased vascular reaction to methyl nicotinate was reported in subjects with sensitive skin (26). Similar analysis can be performed following application of various concentrations of SLS.

Evaluation of Itching Response

Itchy sensation seems to be mediated by a new class of C fibers with an exceptionally lower conduction velocity and insensitivity to mechanical stimuli (27). Indeed, no explanation of the individual susceptibility to the itching sensation without any sign of coexisting dermatitis has been found. Laboratory investigations have also been limited.

An itch response can be experimentally induced by topical or intradermal injections of various substances, such as proteolytic enzymes, mast cell degranulators, and vasoactive agents.

Histamine injection is one of the more common procedures: histamine dihydrochloride (100 μg in 1 mL of normal saline) is injected intradermally in one forearm. Then, after different time intervals, the subject is asked to indicate the intensity of the sensation using a predetermined scale and the duration of itch is recorded. Information is always gained by the subject's self-assessment.

A correlation between whealing and itching response produced by applying a topical 4% histamine base in a group of healthy young females, has been investigated (21). The itching response was graded by the subjects from none to intense. The data showed that the dimensions of the wheals do not correlate with pruritus. Also, itch and sting perception seem to be poorly correlated.

The cumulative lactic acid sting scores were compared with the histamine itch scores in 32 young subjects; all the subjects who were stingers were also moderate-to-intense itchers, while 50% of the moderate itchers showed little or no stinging response (21).

Furthermore, the histamine-induced itch sensation decreases after topically applied aspirin (33). This result can be attributed to the role that prostaglandins play in pain and itch sensation (34).

Localized itching, burning, and stinging can be also a feature of nonimmunologic contact urticaria, a condition characterized by a local wheal and flare after exposure of the skin to certain agents. Non-antibody-mediated release of histamine, prostaglandins, leukotriens, substance P, and other inflammatory mediators may likely be involved in the pathogenesis of this disorder (35). Several substances such as benzoic acid, cinnamic acid, cinnamic aldehyde, and nicotinic acid esters are capable of producing contact nonimmunologic urticaria, eliciting local edema, and erythematous reactions in half of the individuals. Provocative tests are based on an open application of such substances and well reproduce the typical symptoms of the condition.

Washing and Exaggerated Immersion Tests

The aim of these tests is to identify a subpopulation with an increased tendency to produce a skin response.

In the washing test (36), subjects are asked to wash their face with a specific soap or detergent. After washing, individual sensation for tightness, burning, itching, and stinging is evaluated using a point scale, which is previously determined.

The exaggerated immersion test is based on soaking the hands and forearms of the subjects in a solution of anionic surfactants (such as 0.35% paraffin sulfonate, 0.05% sodium laureth sulfate-2EO) at 40°C, for 20 minutes.

After soaking, hands and forearms are rinsed under tap water and patted dry with a paper towel. This procedure is repeated two more times, with a 2-hour period between each soaking, for two consecutive days. Prior to the procedure, baseline skin parameters are evaluated. The other evaluations are taken 2 hours after the third and sixth soaking and 18 hours after the last soaking (recovery assessment). All of the skin parameters are performed after the subjects have rested for at least 30 minutes at 21 ± 1°C.

BIOENGINEERING TESTS

Physiologic changes indicative of sensitive skin can be detected at low levels prior to clinical disease presentation by using noninvasive bioengineering tests.

Transepidermal Water Loss

TEWL is used to evaluate water loss that is not attributed to active sweating from the body through the epidermis to the environment and represents a marker of SC barrier function. TEWL assessment can be performed using different techniques (close chambers method, ventilate chambers method, and open chambers method). Measurements are based on the estimation of water pressure gradient above the skin surface. The open chambers instruments consist of a detachable measuring probe connected by a cable to a portable main signal-processing unit. The probe is provided of chambers open at both ends with relative humidity sensors (hygrosensors) paired with temperature sensors (thermistors). TEWL values ($g/m^2/hr$) are calculated by the signal-processing units in the probe handle and main unit, and digitally displayed. The close chamber instrument consists of a closed cylindrical chamber containing the sensors. The humidity sensor based on a thin-film capacitative sensor integrated to a hand-held microprocessor-controlled electronic unit provided with a digital readout for the TEWL value (37,38).

Corneometry

The corneometry is a method to measure SC water content (electrical measurements). The instrument consists of a probe that should be placed on a hair-free skin surface with slight pressure. It

is described as being a "capacitance" measuring device, operating at low frequency (40–75 MHz), which is sensitive to the relative dielectric constant of material in contact with the electrode surface. It estimates in about 20 ms water content of the SC to an approximate depth ranging between 60 and 100 mm, using arbitrary units.

The presence of salts or ions on the skin surface can affect the reading.

Laser Doppler Velocimetry

A monochromatic light from a helium–neon laser is transmitted through optical fibers to the skin. The light is reflected with Doppler-shifted frequencies from the moving blood cells in the upper dermis at the depth of ~1 mm. The LDV extracts the frequency-shifted signal and derives an output proportional to the blood flow. LDV is useful to evaluate the degree of skin irritation (39).

Colorimetry

Surface color may be quantified using the Commission Internationale de L'Eclairage (CIE) system of tristimulus values. The device utilizes silicon photocells. The measuring head of these units contains a high-power pulsed xenon arc lamp, which provides two CIE illuminant standards. The color is expressed in a three-dimensional space. The coordinates are expressed as L* (brightness), a* value (color range from green to red), and b* value (color range from blue and yellow). The a* value, related to skin erythema, increases in relation to irritation and skin damage.

Corneosurfametry

This method (40) investigates the interaction of surfactants with the human SC. It is performed as follows: cyanoacrilate skin surface stripping (CSSS) is taken from the volar aspect of the forearm and sprayed with the surfactant to be tested. After 2 hours the sample is rinsed with tap water and stained with basic fuchsin and toluidine blue dyes for 3 minutes. After rinsing and drying, the sample is placed on a white reference plate and measured by reflectance colorimetry (Chroma Meter@ CR200, Minolta, Osaka, Japan).

The index of redness (CIM = Luminacy L* – Chroma C*) is taken as a parameter of the irritation caused by the surfactant. This index has a value of 68 ± 4 when water alone is sprayed on the sample and decreases when surfactant is tested, with stronger surfactants lowering the values.

Piérard (41), testing different shampoo formulations in volunteers with sensitive skin, demonstrated that corneosurfametry correlates well with *in vivo* testing. A significant negative correlation ($P < 0.001$) was found between values of colorimetric index of mildness (CIM) and the skin compatibility parameters that include a global evaluation of the colorimetric erythemal index, and the TEWL differential, both expressed in the same order of magnitude.

In the same study corneosurfametry showed less interindividual variability than *in vivo* testing, allowing a better discrimination among mild products.

An interesting finding showed that sensitive skin is not a single condition. Goffin (42) hypothesized that the response of the SC to an environmental threat might be impaired in different groups of subjects experiencing sensitive skin. Data of the corneosurfametry performed after testing eight different house-cleaning products, showed that the overall SC reactivity, as calculated by the average values of the corneosurfametry index and the CIM, is significantly different ($P < 0.01$) between detergent-sensitive skin and both nonsensitive and climate/fabric-sensitive skin.

Irregularity Skin Index

Irregularity skin index (ISI) can contribute to the identification of subjects with sensitive skin.

In a recent study (43) conducted on 243 subjects positive to the lactic acid stinging test, slides of CSSS, obtained from the volar aspect of the forearm, were examined by means of a computer-assisted fast Fourier transform to determine the skin surface microrelief. Acquisition of the images was performed by a stereomicroscope connected to an analogic video camera. The results confirmed a significant correlation ($P < 0.001$) between intensity of symptoms in "stingers" and ISI. This procedure represents a valuable and promising tool for the study and the diagnosis of sensitive skin.

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54 Dermatotoxicology of specialized epithelia: Adapting cutaneous test methods to assess topical effects on the vulva

Miranda A. Farage and Howard I. Maibach

Investigating cutaneous effects is a fundamental step in assessing the safety of topical products. The conventional arsenal for evaluating cutaneous effects includes single- and multiple-exposure patch tests for skin irritation as well as repeat insult patch tests for contact sensitization. However, these standard patch test methodologies, which were designed to assess skin effects at exposed or partially occluded areas of the anatomy, are not optimized for evaluating topical reactions in specialized epithelia, such as the vulva. The vulva differs from skin at other sites in morphology and regional differentiation (1), tissue structure (2,3), blood flow (4), occlusion (5), and tissue hydration (5,6), factors which may in turn influence its susceptibility to topically applied agents (7–10). Moreover, certain subpopulations, such as older women, may be more susceptible to vulvar injury due to postmenopausal vulvar atrophy and the increased prevalence of urinary incontinence (11,12); indeed, genital skin sensitivity is more likely to be reported by 50 years or older people (13,14). This review compares the characteristics of vulvar epithelia to skin at other sites (Table 54.1) and describes how cutaneous test methods are being adapted to better assess the potential effects on vulvar tissue.

VULVAR ANATOMY AND REGIONAL DIFFERENCES IN TISSUE STRUCTURE

Figure 54.1 illustrates vulvar anatomic features. The vulva is bordered anteriorly by the mons pubis, a mound of tissue bearing a characteristic triangular conformation of pubic hair; posteriorly by the perineum, which separates the vulva from the anus; and laterally by the labiocrural folds, which separate the vulva from the upper thighs. The labia majora, lobes that lie medial to the labiocrural folds, enclose the thinner labia minora. The labia minora surround the interior portion of the vulva, which comprises the vulvar vestibule and the edge of the hymen at the vaginal orifice (introitus). The urethral orifice is anterior to the introitus. The labia minora join anteriorly to the urethral orifice to form the preputium clitoridis, a hood of tissue that covers the clitoris. The posterior junction of the labia minora forms the fourchette. The anterior and posterior commissures are located at the junctures of the labia majora anterior to the clitoris and posterior to the fourchette, respectively.

Being derived from two distinct embryonic layers, the ectoderm and the endoderm, vulvar tissue displays regional differences in morphology and structure. Like skin at other sites, the cutaneous epithelium of the mons pubis, labia majora, and perineum is derived from the embryonic ectoderm. It exhibits a keratinized squamous structure with sweat glands, sebaceous glands, and hair follicles (Fig. 54.2A). However, the thickness and degree of keratinization of vulvar skin decreases in moving from the labia majora to the surface of the clitoris and the labia minora. The epithelium of the labia minora is markedly thinner than that of the labia majora and bears no sweat glands or hair follicles in women of reproductive age (3).

From approximately the inner third of the labia minora to the introitus, the epithelium becomes nonkeratinized (Fig. 54.2B). Hart's line, which demarcates the junction of keratinized skin and nonkeratinized tissue, borders the vulvar vestibule. The nonkeratinized vulvar vestibule is derived from the embryonic endoderm. Its epithelial structure histologically resembles that of the vagina and the nonkeratinized regions of the oral cavity (2,15). The superficial stratum bears large, moderately flattened cells that lack keratin but contain glycogen granules and frequently pyknotic nuclei. Beneath this stratum, differentiation of the inner mucosal cells is indistinct: polyhedral cells migrate upward from the generative basal layer, but remain loosely packed and do not form clearly demarcated substrata as observed in the skin. Cervicovaginal secretions moisten the vulvar vestibule.

IMMUNE CELL POPULATIONS AND RESPONSIVENESS

The vulvar epithelium is an immunocompetent tissue. Langerhans cells are the most common immune cell type in the vulva; intraepithelial and perivascular lymphocytes are infrequently found (16). Langerhans cells serve as sentinels: they sample antigen that crosses the tissue and present it to T cells in the lymph nodes, initiating the delayed contact hypersensitivity response. No difference in Langerhans cell densities exists between keratinized and nonkeratinized vulvar tissue (16).

Menstrual cycle variability in vulvar immune cell populations has not been studied directly; however, cyclical variability is not expected, as the number and distribution of immune cells in the vagina, a hormonally responsive tissue, remain stable throughout the menstrual cycle (17).

Although different regions of the vulva have similar population densities of Langerhans cells, vulvar skin and mucosa may exhibit distinct responses to antigen. Skin exposure to antigens can result

TABLE 54.1
Comparison of the Skin and Vulvar Epithelia

		Vulva	
Characteristic	**Exposed Skin**	***Keratinized Epithelium***	***Nonkeratinized Mucosa***
Epithelial structure	Keratinized, squamous epithelium with hair follicles, sweat glands, and sebaceous glands Regional variations in thickness	*Mons pubis, labia majora:* Keratinized epithelium with hair follicles, sweat glands, and sebaceous glands (3) *Outer two-thirds of labia minora:* Thinner, keratinized epithelium lacking hair follicles and sweat glands (2,3)	*Inner third of the labia minora and vestibule:* Thin, nonkeratinized mucosal epithelium comparable in structure to buccal and vaginal mucosae (2,15)
Langerhans cell densities	Langerhans cell densities range from 400–1000 cells/mm^2 of skin (79)	Langerhans cell densities similar to the skin (16)	No difference in Langerhans cell densities between keratinized and nonkeratinized regions (16) Menstrual cycle unlikely to have an impact (17)
Occlusion	Occurs at certain sites, e.g., axilla.	Anatomic and garment-related occlusion	Anatomical occlusion
Friction	Varies by anatomic site	Higher friction coefficient than forearm skin (27)	Not determined
Hydration	Varies by anatomic site	More hydrated than exposed skin, based on transepidermal water loss (5,26)	Hydrated by cervicovaginal secretions
Permeability	A function of skin thickness and concentration of hair follicles, sweat glands, and sebaceous glands	Greater occlusion and hydration may affect permeability relative to exposed sites, depending on nature of applied vehicle and penetrants. Sevenfolds more permeable to hydrocortisone than forearm skin (30)	More permeable than keratinized skin; comparable to buccal mucosa.(35,36) Characteristics of tissue structure (2,3), lipid profile (39,40), thickness, hydration, and occlusion lead to increased permeability

Source: From Ref. 80.

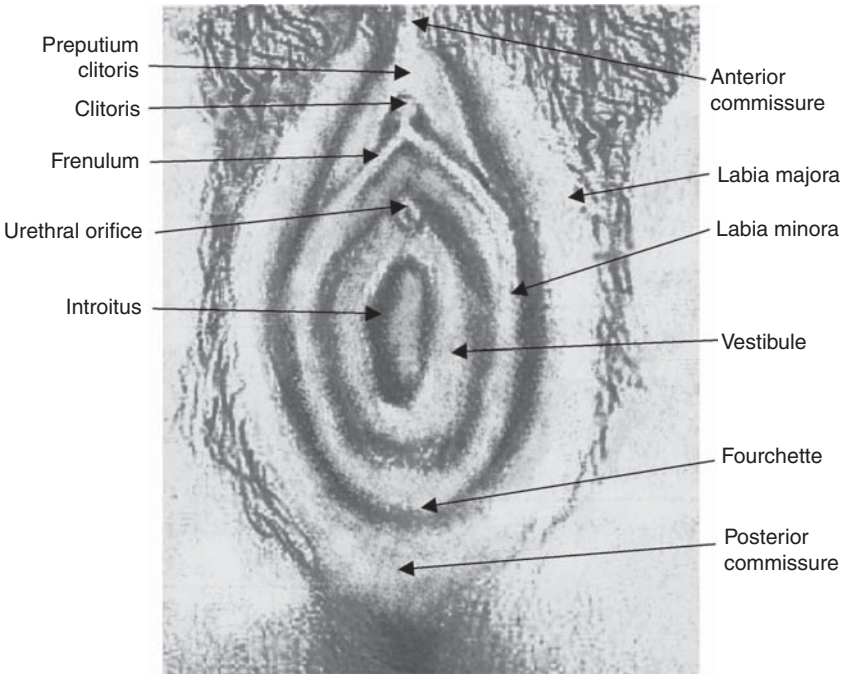

FIGURE 54.1 Anatomy of the vulva. *Source*: From Ref. 80.

in sensitization; indeed, allergic contact dermatitis to topical agents is a prime contributor to persistent vulvar discomfort (18–20). However, in the nonkeratinized mucosa, the possibility exists that antigen exposure may induce tolerance. Tolerance induction, best characterized in the oral mucosa, is not related to the phenotype of resident Langerhans cells, but results from altered responses at the level of the draining lymph nodes (21,22). Although this phenomenon has not been studied in the mucosa of the vulvar vestibule, tolerance induction has been demonstrated in vaginal tissue of animal models, where the phenomenon is hormonally regulated (23).

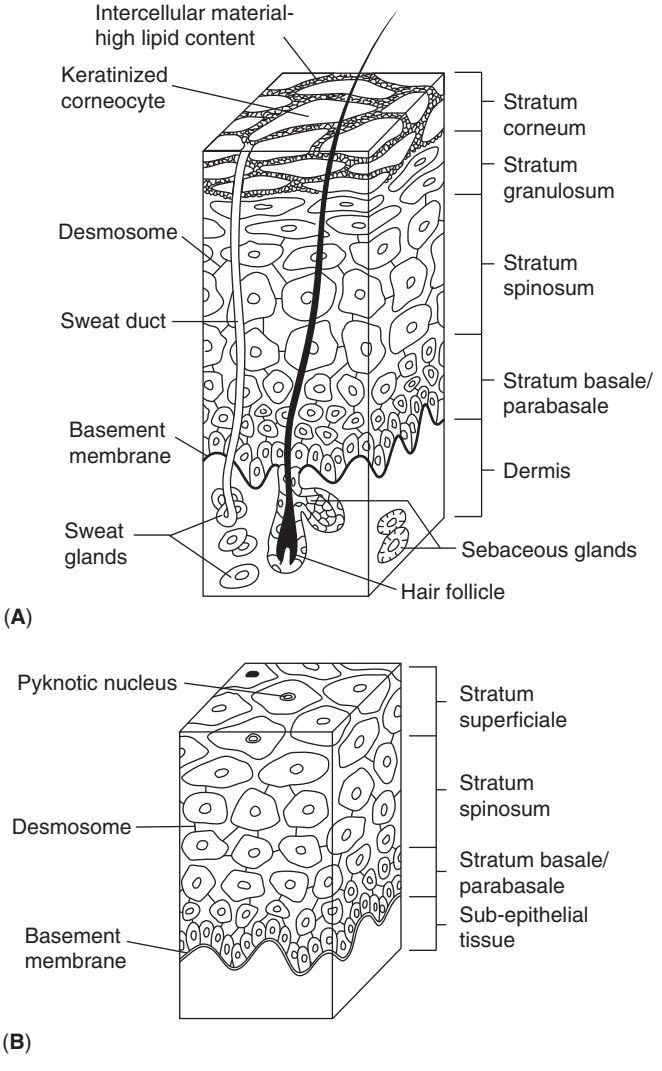

FIGURE 54.2 Structure of vulvar epithelia. (**A**) Keratinized vulvar skin; (**B**) nonkeratinized vulvar vestibule. *Source*: From Ref. 80.

In mice, vaginally induced tolerance occurred only during the estrogen-dominant phase of the estrus cycle when sperm exposure would occur. This protective mechanism may limit the response to antigens in sperm.

An article reviews the components of innate immunity that are functional in the female cervicovaginal environment (24).

BLOOD FLOW, TISSUE HYDRATION, AND OCCLUSION

Vulvar skin has higher levels of blood flow, tissue hydration, and occlusion than exposed skin (Table 54.2). Blood flow in the skin of the labia majora is over twice that in forearm skin (25). Moreover, histamine treatment increases the blood flow in vulvar skin at doses to which forearm skin is unresponsive (4). Vulvar skin is more hydrated than exposed skin and exhibits reduced water barrier function. Specifically, measurements of transepidermal water loss (TEWL) demonstrate that water diffuses across the stratum corneum (SC) of the labia majora faster than across the SC of the forearm (6,26). This reflects, in part, elevated vulvar skin hydration from occlusion. But vulvar skin also presents an intrinsically lower barrier to water loss: steady-state TEWL values remain higher on the labia majora than on the forearm even after equilibration with the environment or after the prolonged drying of both sites with a desiccant (6,7). Furthermore, the comparatively greater hydration of occluded vulvar skin raises its friction coefficient (Table 54.2), which may make vulvar skin more susceptible to mechanical damage (27).

PERMEABILITY AND SUSCEPTIBILITY TO IRRITANTS

Predicting tissue permeability is complex. The phenomenon depends on three principal factors: the extent to which the penetrant partitions into the tissue, the rate at which the penetrant diffuses through the tissue, and the distance to be traversed (28). The extent to which an exogenous agent partitions into and diffuses through vulvar tissue will depend on the physicochemical characteristics of the penetrants and the nature of the applied vehicle but will also be affected by regional differences in vulvar epithelial structure, lipid composition, and hydration. In addition, regional differences in the thickness of the vulvar epithelium will alter the distance a penetrant must traverse at different sites. A consideration of these characteristics, discussed in detail below, indicate that a conservative approach to evaluating the safety of topically applied materials is warranted.

Keratinized Labia Majora Skin

The keratinized skin of the labia majora exhibits variable permeability to exogenous agents compared with exposed forearm skin, in some instances higher and in others lower (Table 54.2). For example, the skin of the labia majora is substantially more permeable to hydrocortisone than the skin of the forearm (29,30). Contributing factors may include the elevated hydration of vulvar skin relative to forearm skin, its higher concentration of hair follicles and sweat glands, and its elevated cutaneous blood flow.

However, tissue penetration rates depend not only on skin characteristics but also on the properties of the penetrant. For example, no difference was found in the rate of testosterone penetration through vulvar and forearm skin, although the skin at both sites was far more permeable to testosterone than to hydrocortisone (Table 54.2) (30). The rapid penetration of testosterone through both vulvar and forearm skin may be related to its hydrophobicity as well as to the presence of androgen receptors at both anatomic sites (31).

Similarly, vulvar skin susceptibility to topical irritants varies. Heightened vulvar skin hydration may influence penetration rates of polar irritants. Skin penetration of a polar agent depends on its external concentration and its solubility in the applied medium relative to skin tissue (32,33). Because the SC is lipophilic, penetration of polar or charged substances is usually disfavored; however, the elevated hydration of the vulvar SC could facilitate entry of polar irritants. Consistent with this hypothesis, concentrated aqueous solutions of the polar irritants, benzalkonium chloride, and maleic acid irritated vulvar skin more than forearm skin (10).

By contrast, vulvar skin was less affected than forearm skin by the model irritant, sodium lauryl sulfate (SLS), applied as a dilute aqueous solution (7,9). In this instance, tissue irritation may have been affected by the physicochemical properties of the surfactant as well as its efficient solvation at low concentration by the aqueous medium. Specifically, surfactants bear both a charged, polar head and a lipophilic tail. Skin penetration of the charged head is disfavored; in dilute aqueous solution, solvation of the polar head would be difficult to overcome. Consequently, the partitioning of the hydrophobic surfactant tail into SC lipids may have served as

the principal driving force for its irritant effects, a phenomenon that would be favored on less hydrated forearm skin.

Interestingly, vulvar skin is relatively insensitive to menses-induced irritation (34). In a 48-hour occlusive skin patch test on the labia majora and upper arm, menses and venous blood elicited mild skin erythema on the upper arm only; no discernible reaction occurred on the labia majora. This insensitivity may represent an adaptation to menstruation.

In summary, keratinized vulvar skin varies in its susceptibility to topical penetrants and irritants when compared with forearm skin. Although the comparative permeability of vulvar skin depends on a combination of factors, in several instances vulvar skin is more susceptible to topical agents than the skin at other sites. Vulvar skin also has an elevated friction coefficient (8), which may contribute to breaches in skin integrity. Moreover, when obesity, impaired mobility, or urinary incontinence exist, friction and chaffing, shear forces, and excess skin hydration may compromise vulvar skin integrity. Taken together, these considerations support a conservative approach to assessing the potential effects of topical products on vulvar skin.

Nonkeratinized Mucosa of the Vulvar Vestibule

Nonkeratinized epithelia generally are more permeable to external penetrants than the skin, a critical consideration for the safety of topical materials. The heightened permeability of nonkeratinized epithelia has been documented by studies on oral tissue, which, like the vulva, displays regional differences in structure and keratinization. The nonkeratinized buccal mucosa and the thinner nonkeratinized mucosa of the floor of the mouth are 10- and 20-fold more permeable to water, respectively, than keratinized skin (35). Buccal mucosa is also more permeable than the skin to horseradish peroxidase, although absolute penetration rates of this large molecule are lower than those of water (36).

The heightened permeability of nonkeratinized tissue results from several factors. First, the absence of an SC removes a principal barrier to entry of external agents. Second, the more loosely packed cell layers create a structure with less resistance to paracellular movement, the principal route by which most penetrants traverse tissues (37,38). Third, such tissues have a less-structured lipid barrier with lower resistance to molecular diffusion (39,40). Fourth, thinner epithelia (such as the buccal mucosa and vulvar vestibule) present a shorter path length to be traversed.

Nonkeratinized tissue is also more vulnerable to breaches in tissue integrity, which can augment tissue penetration. For example, buccal tissue was 40-fold more permeable than keratinized skin to the organic base, nicotine, an irritant that increases the penetration of coadministered compounds (41,42).

The heightened permeability of the vulvar vestibule may be inferred from studies on vaginal and buccal epithelia, which serve as surrogate

TABLE 54.2
Quantitative Comparison of Biophysical Variables, Permeability and Irritant Susceptibilities in Forearm and Vulvar Skin (Labia Majora)

Parameter Assessed (units)	Forearm	Vulva	Statistical Significance (n = number of subjects)	Reference
Transepidermal water loss (g/m^2h)	3.5 ± 0.3	14.5 ± 1.3	$P < 0.001$[a] ($n = 44$)	(27)
Friction coefficient (unitless)	0.48 ± 0.01	0.66 ± 0.03	$P < 0.001$[a] ($n = 44$)	(27)
Blood flow (Absorbance units)	22.0 ± 3.0	59.5 ± 7.4	$P < 0.001$[a] ($n = 9$)	(4)
Hydrocortisone penetration (% of applied dose absorbed in 24 hr)	2.8 ± 2.4	8.1 ± 4.1	$P < 0.01$[b] ($n = 9$)	(30)
Testosterone penetration (% of applied dose absorbed in 24 hr)	20.2 ± 8.1	25.2 ± 6.8	NS[b,c] ($n = 9$)	(30)
Frequency of irritant reactions to 20% maleic acid solution (%)	62	76	— ($n = 21$)	(10)
Mean intensity of irritant reactions to 20% maleic acid at 24 hr postapplication (0–3 visual scale)	0.86 ± 0.36	1.29 ± 0.83	$P = 0.036$[a] ($n = 21$)	(10)
Frequency of irritant reactions to 17% benzalkonium chloride solution (%)	9	57	Not determined ($n = 21$)	(10)
Mean intensity of irritant reactions to 17% benzalkonium chloride solution at 24 hr postapplication (0–3 visual scale)	0.19 ± 0.33	1.00 ± 0.88	$P = 0.0003$[a] ($n = 21$)	(10)
Irritant reactions to 1% sodium lauryl sulfate at day 2 postapplication (proportion of scores > 1 on 0–4 scale)	9/10	0/10	$P < 0.05$[d] ($n = 10$)	(9)

[a]Student's *t* test.
[b]One-way analysis of variance followed by Newman–Keuls multiple range test.
[c]Not significant.
[d]Wald–Wolfowitz two-sample test.
Source: From Ref. 80.

tissues. Vaginal and buccal epithelia have similar ultrastructural features and lipid composition (15). Comparable tissue penetration rates at steady-state have been observed in both tissues for a range of model penetrants, including water, estradiol, vasopressin, and low–molecular weight dextrans (43–46). Like these epithelia, the thin, nonkeratinized vulvar vestibule is expected to be more permeable and more vulnerable to topical agents than keratinized skin.

ADAPTING CUTANEOUS TEST METHODS TO ASSESS TOPICAL EFFECTS ON THE VULVA

As evidenced above, vulvar tissue may be more susceptible to the effects of topical agents than exposed skin because of the differences in tissue structure and physiologic characteristics. This demands a more conservative approach to the safety assessment of topical formulations and products. Because it is impractical to conduct routine predictive testing on the vulva, our laboratories have adapted standard methodologies and developed new approaches to cutaneous testing to aid in the risk assessment of topical vulvar exposures (47). Several avenues have been pursued (Table 54.3):

1. A more conservative quantitative risk assessment (QRA) for induction of allergic contact dermatitis has been developed for mucosal exposures and a modified human repeat insult patch test (HRIPT) protocol with potentially heightened sensitivity can be employed to evaluate

TABLE 54.3
Protocols and Analytic Techniques Adapted for Assessing Topical Effects of Articles or Formulations that Contact the Vulva

Endpoint of Interest	Standard Protocol for Cutaneous Safety Assessment	Modified Protocol Adapted for Mucosal Safety Assessment
Induction of delayed contact hypersensitivity	HRIPT (61) A traditional protocol employs a 3-wk induction phase of 9, 24-hr applications with 24-hr rest periods between applications (48 hr on weekends). Between 100 and 200 subjects are typically evaluated.	Modified HRIPT (59) The modified HRIPT protocol increases the number of applications during the induction phase to 15 (consecutive 24-hr applications, 5 days/wk for 3 wk, with 48-hr rest periods on weekends). The cumulative exposure duration rises by 67% relative to the traditional protocol.
Chemical and frictional irritation	Fabric wear test of laundered garments (wristbands, men's briefs, undershirts) to evaluate the safety of laundry products. Treated samples are worn for up to 2 wk. Skin reactions are scored for erythema and dryness.	BTK test for feminine hygiene products (64) Test materials are applied daily to the popliteal fossa under an occlusive elastic bandage and worn for 6 hr/day for 4–5 days. Skin reactions are scored for erythema and dryness. The technique exaggerates exposure conditions and increases test sensitivity.
Chemical and frictional irritation from repeated wiping	FCAT Test samples are wiped repeatedly on the volar skin of the forearm (15–30 sec, 4 times/day for 3–5 days)	Modified FCAT (69) Sample application lengthened to 6–8 consecutive days. Conditions pertinent to vulvar exposures can be simulated (pretreatment of the application site by hydrating the skin overnight with a fluid-soaked bandage or pretreatment of the application site with mild surfactant or by tape stripping to compromise the skin barrier)
Cumulative irritation	Cumulative irritation patch test Test sample is applied repeatedly to the same location via open or semi-occluded patch for 14–21 consecutive days. Skin reactions are scored for erythema and dryness.	Cumulative irritation patch test in people with sensitive skin (68) Test performed in subjects who claim to have sensitive skin and have a history of adverse reactions to everyday products. Potentially may increase test sensitivity if an appropriate subpopulation can be identified.
Detection of tissue inflammation	Standard visual scoring by trained personnel Trained personnel score erythema and tissue dryness with the unaided eye or by colposcopy using standard illumination.	Enhanced visual scoring with parallel polarized and cross-polarized light (51) Illumination with cross-polarized light (Syris V600™ system enables visualization of subsurface inflammation to a depth of approx. 1 mm. Technique increases the ability to detect subclinical inflammation not visible with standard illumination. Measurement of molecular markers of inflammation (53) Immunoassays of cytokine mediators of inflammation (captured noninvasively with tape applied to the epithelial surface) allow inflammatory changes to be detected before they are visually apparent.
Analysis of subjective sensory effects	Questionnaires administered in prospective product trials Questionnaires on the nature, frequency, and severity of sensory effects (e.g., burning, itching, pain, chaffing, sticky feeling, and so on) provide additional perspective on the product use experience not gained by objective dermatologic examination.	Questionnaires administered in predictive tests adapted for evaluating specialized epithelia (e.g., BTK test with visualization of subsurface inflammation). The frequency of certain subjective effects (e.g., burning) reported in premarket predictive testing, corroborated by visualization with subclinical inflammation, shows promise for identifying subtle differences in the tolerability of products that contact the vulva

Abbreviations: BTK, behind-the-knee; FCAT, forearm controlled application test; HRIPT, human repeat insult patch test.

materials that contact vulvar tissue. These adaptations increase the margin of safety for assessing the risk of contact sensitization.

2. Newer, more conservative methodologies have been developed to assess the combination of chemical irritation and friction pertinent to some topical vulvar exposures. The behind-the-knee (BTK) clinical test protocol (a global American Society Test Material [ASTM] protocol in which test articles are applied to the skin of the popliteal fossa under an elastic bandage) has been validated for evaluating articles such as sanitary pads and tampons, for which intimate contact, occlusion, movement, and/or friction may contribute to topical vulvar effects (48–50). A modified forearm controlled application test (mFCAT) has been developed to assess the potential for cumulative skin irritation from repeated, intermittent exposure to mild articles, such as toilet tissue, feminine wipes, and baby wipes.
3. Sensitive techniques are being implemented to better discern clinical and subclinical inflammation. Techniques such as tissue visualization with parallel-polarized and cross-polarized light (51,52) and the detection of molecular markers of inflammation, such as cytokines (53), should lower the detection limit of inflammatory changes.
4. Subjective reports of sensory discomfort can be analyzed to potentially discriminate between mild materials that exhibit similar cutaneous effects by visual inspection but still differ in consumer preference or tolerability (54).

The predictive nature of these new or modified approaches is being validated by correlating the test results on specific products or formulations with observations from prospective clinical trials of the same products and formulations under normal conditions of use. These strategies result in a more rigorous safety assessment of products and formulations intended to contact vulvar tissue. They are described in detail in Sections "Assessment of the risk of induction of allergic contact dermatitis" through "Evaluation of subjective sensory effects."

Assessment of the Risk of Induction of Allergic Contact Dermatitis

The potentially heightened permeability of vulvar tissue to topically applied agents has direct bearing on the risk of induction of allergic contact dermatitis. In order for the induction of allergic contact dermatitis to occur, externally applied contact allergens must first cross the tissue surface for the antigen to become available to resident Langerhans cells. Because most data on contact allergy derive from exposure to skin at other sites, extrapolating to vulvar exposures requires an extra measure of conservatism to account for tissue permeability differences.

To address this, two approaches have been employed. First, a higher margin of safety has been incorporated into the QRA process for the induction of allergic contact dermatitis. In brief, QRA is a systematic method for estimating the health risk of chemicals that cause dose-dependent, threshold effects (55). The process compares the estimated exposure to a potential contact allergen resulting from product use to a safe reference value (56) derived from an experimentally or clinically determined sensitization induction threshold. To derive the reference value, the experimental threshold dose is divided by sensitization uncertainty factors that account for the need to extrapolate from experimental exposure conditions to the characteristics of actual consumer exposure (55,57). We proposed the use of uncertainty factors in the range of 1–10 for extrapolating from exposed skin to vulvar skin and 1–20 for extrapolating from exposed skin to mucosal tissues (Table 54.4). These factors are greater than those typically applied to exposure at other anatomic sites. The scientific rationale for the ranges is based principally on permeability differences between exposed skin and vulvar tissues, and has been delineated in detail elsewhere (58).

Second, a modified protocol for the HRIPT has been proposed to assess materials that contact the vulva (59). The HRIPT is a clinical patch test for assessing the potential induction of allergic contact dermatitis (60). This test is not used for hazard assessment but may be performed after the QRA to further substantiate that the risk of inducing allergic contact dermatitis is negligible. One traditional protocol, optimized for exposure to keratinized skin, employs a 3-week induction phase of 9, 24-hour applications with 24-hour rest periods (48 hours on weekends) (61). Between 100 and 200 subjects are typically evaluated. In 1945, Henderson and Riley (62) discussed the predictive power of extrapolating from a small test population to a large exposed population based on statistical considerations. Assuming there exists a fraction ***p*** in the population who would become sensitized, the probability that one

TABLE 54.4
Quantitative Risk Assessment Uncertainty Factors for Topical Mucosal Exposures to Potential Contact Allergens

Product Type	Uncertainty Factor	Rationale
Personal hygiene products. Conventional sanitary napkins, incontinence pads	1–10	Contact is predominantly with stratified, squamous keratinized epithelium. The default uncertainty factor range (for differences in body site, skin integrity, and occlusion) applies
Oral care products Dentifrice, mouthwash, chewing gum	1–10	Contact is with a mixture of keratinized and nonkeratinized tissue For many products, rapid dispersion, limited contact time, and salivary dilution occurs make the lower end of the range more relevant
Personal hygiene products Tampons, interlabial pads, and others Oral care products Denture adhesives, overnight tooth whiteners, and others	1–20	Close, occluded contact with nonkeratinized mucosa may occur for extended periods. Nonkeratinized oral and vulvovaginal mucosae are similar in structure and more permeable to molecules. The high end of the range may be applicable

Source: From Ref. 58.

or more of ***n*** independent subjects will exhibit a response is given by a binomial distribution:

$$1 - (1 - p)^n$$

This predicts that if 5% of the population can be sensitized, the probability that at least one subject will respond in a test of 200 people is greater than 99%. The smaller the proportion of potential respondents, the lower the probability of detection. For example, if potential respondents represent 1% of the population, the probability of detection falls to 87%.

Our objective was to increase test sensitivity for extrapolating to mucosal exposures while maintaining reasonable and practical group sizes. Kligman's pioneering studies demonstrated that induction rates are a function of the dose as well as the number, duration, and spacing of exposures (63). As it is not always feasible to increase the applied dose (particularly when testing solid articles), the modified HRIPT protocol we proposed employs daily, 24-hour applications, 5 days per week, during the induction phase (59). Consequently, the number of applications increases to 15 and the cumulative exposure duration rises by 67% relative to the traditional protocol. This approach should increase the cumulative exposure dose during the induction period in situations where penetration is more rapid, as is expected to occur in mucosal tissue. Another advantage of the proposed protocol is that it incorporates three repetitions of a five-application induction course while maintaining rest periods. Kligman demonstrated that although continuous exposure during the induction phase is less effective at induction than allowing rest periods, three repetitions of a five-application induction course increased sensitivity to near-threshold concentrations of allergen (63). Finally, the pattern of consecutive daily exposure in the proposed protocol is more representative of the way consumers use feminine hygiene products. The proposed advantages of this modified protocol are based on theoretic considerations. To further validate this approach, comparative studies of the traditional and modified protocols are planned.

The Behind-the-Knee Clinical Test for Assessing Chemical and Mechanical Irritation

The BTK clinical test method was developed to assess the combination of chemical irritation and frictional effects by means of repeated topical application of test materials to the popliteal fossa (48,49). The method is principally applied to solid articles (such as sanitary pads, uncompressed tampons, infant diapers, and adult incontinence products) that intimately contact the vulva under occluded conditions.

Materials are applied to the skin of the popliteal fossa (the diamond-shaped area behind the knee joint) for 6 hours daily for four consecutive days, and held in place with an elastic bandage. Visual skin grading is performed daily, using standard scoring scales for erythema and tissue dryness, at two time points: 30 minutes after test material removal and after an overnight (18-hour) recovery period. The frequency of subjective reports of sensory irritation is also documented daily.

The BTK clinical test was designed to be a comparative toxicity test to evaluate a novel material relative to a reference material with a well-established safety profile (Fig. 54.3). The test material is applied to one leg and the reference material or control to the other. This protocol also allows comparative testing of different products or different conditions (such as using wet and dry samples, hydrating the skin by wet occlusive patch prior to sample application, or sample application to intact and compromised skin). Such test conditions help assess the impact of excess skin hydration or breaches in skin integrity that may be pertinent to infant dermatitis, adult incontinence dermatitis, intertrigo, and so on.

Dozens of materials have been evaluated in the BTK clinical test, including diaper and sanitary pad surface sheets, interlabial pads, panty liners, tampons, fabrics and textiles, and lotion coatings on a solid substrate. The test successfully discriminates between materials that are physically irritating due to friction and those that are not (48). In one series of validation studies, the BTK clinical test successfully distinguished the mechanical irritation potential of three commercially available sanitary pads that were expected to differ in their surface properties (49).

Importantly, the predictive nature of the test has been established by correlating the results of paired product comparisons in BTK clinical test with the results of in-use, crossover trials of the same products. In a series of validation studies, the comparative effects of two menstrual pads, two tampons, and an interlabial pad and panty liner were evaluated in both the BTK clinical test and in prospective trials of the same products under normal conditions of use. In all cases, the BTK clinical test and the product use trials resulted in the same conclusion (64).

Although the BTK clinical test protocol was developed to assess cutaneous effects, validation studies have established its utility for predicting inflammation of the vulvovaginal mucosa (50). In an investigation of the comparative effects of two tampons, the products were first evaluated with the BTK protocol (using uncompressed tampons to ensure intimate skin contact) and the same products were tested in a crossover trial during two consecutive menstrual cycles. In the BTK study, the skin of the popliteal fossa was scored for visually discernible erythema at two time points: 30 minutes after test material removal (limited recovery) and after an 18-hour recovery period. In the crossover trial, six sites (labia minora, introitus, lower and middle vaginal walls, upper vagina, and cervix) were scored by colposcopic examination 3–48 hours following removal of the last tampon used for menstrual protection. Both protocols indicated no significant difference in irritant potential between the products. In the prospective trial, tissue erythema following product use was low at all genital sites, the

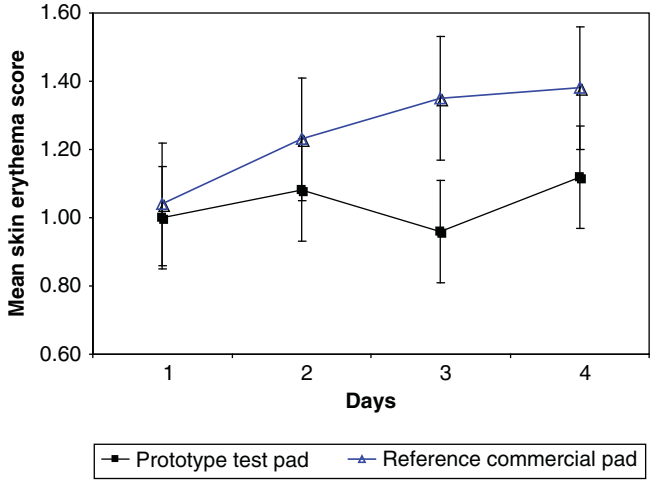

FIGURE 54.3 Comparison of erythema associated with test and control sanitary pads following repeated application to the popliteal fossa ("behind-the-knee" test). *Source*: From Ref. 66.

highest levels being observed on the labia minora (mean scores were 0.26 ± 0.05 and 0.5 ± 0.06 for test and control tampons, where a score of 0.5 is barely discernible erythema) (Table 54.5). By comparison, in the BTK test, erythema scores after an 18-hour recovery period following the final exposure of the 4-day test (0.24 ± 0.06 for both products) approached the erythema levels observed colposcopically on the labia minora and exceeded the levels of introital and vaginal mucosal erythema following menstrual use of the tampons. Most notably, when the recovery time was limited to about 30 minutes, the scores observed in the BTK clinical test after the fourth exposure were 5- to 13-fold higher than those observed following menstrual product use, depending on the anatomic site examined. These observations underscore the heightened sensitivity of the BTK test method and its utility for conservatively predicting both cutaneous and mucosal inflammatory effects.

Beyond its sensitivity and predictive utility, the BTK clinical test offers several practical advantages. It is inexpensive, rapid, and minimally invasive or disruptive to study participants' routine. Unlike in-use testing, it allows simultaneous product comparisons and does not require recruiting menstruating women or people with incontinence. The BTK can also be used in lieu of standard short-term patch tests for skin irritation (typically four consecutive 24-hour patch applications) because the exposure time in the BTK clinical test can be shortened to two, consecutive, 6-hour applications while yielding equivalent results (65). The BTK protocol also can be used in investigative studies to capture and quantify the transfer of chemicals from the surface of a test material to the skin (66). Such information can be employed to estimate exposure for QRA or to assess the delivery of beneficial substances, such skin moisturizers and ointment. The BTK clinical test is an American Society Test Material (ASTM) global standard protocol for evaluating the skin irritation response to products and materials that come into repeated or extended contact with skin (67). Our laboratory now routinely employs the BTK test as part of the premarket safety evaluation of prototype products (66,68); see also chapter 52 of this book).

The Modified Forearm Controlled Application Test

The mFCAT assesses cumulative irritation induced by intermittent, repeated friction with very mild products, such as disposable baby wipes and feminine wipes (69). The test conditions exaggerate exposure sufficiently to enable the discrimination of small but statistically significant differences in skin inflammation or dryness using a reasonable number of subjects. Up to four samples are tested in 60–80 women on adjacent application sites on the volar forearm (women are preferred because they have minimal hair on the volar forearm, the presence of which makes visual scoring of the skin difficult). The test period is 6–8 consecutive days. The wipes to be evaluated are folded several times and then applied to the designated forearm site by wiping with light pressure, four times each day, 1½ hours apart, repeatedly for 15 seconds for the first three applications and then for 40 seconds for the final application of the day. Skin erythema and dryness are scored visually

Table 54.5
Comparison of Tissue Erythema Scores for Control and Experimental Tampons in the BTK Test and in a Prospective Trial of the Products for Menstrual Use

	Experimental Tampon			Control Tampon			Experimental *Vs* Control
	Mean	±	SE	Mean	±	SE	*P* value
BTK Study (*n* = 17)							
Baseline							
Day 1 AM	0.00	±	0.00	0.00	±	0.00	
Scores upon product removal							
Day 1 PM	1.12	±	0.09	1.12	±	0.11	1.000
Day 2 PM	1.27	±	0.11	1.24	±	0.09	0.739
Day 3 PM	1.44	±	0.08	1.44	±	0.08	1.000
Day 4 PM	1.18	±	0.09	1.27	±	0.09	0.257
Scores following overnight recovery							
Day 2 AM	0.15	±	0.06	0.18	±	0.06	0.564
Day 3 AM	0.18	±	0.06	0.18	±	0.06	1.000
Day 4 AM	0.24	±	0.06	0.24	±	0.06	1.000
In-use study (*n* = 43)							
Labia minora	0.36	±	0.05	0.50	±	0.06	0.958
Introitus	0.13	±	0.03	0.26	±	0.05	0.983
Lower vaginal wall	0.08	±	0.03	0.20	±	0.04	0.996
Middle vaginal wall	0.10	±	0.03	0.17	±	0.04	0.946
Upper vagina	0.09	±	0.03	0.20	±	0.04	0.994
Cervix	0.17	±	0.04	0.24	±	0.05	0.910

Treatment comparisons in the BTK test at each scoring time point were performed using stratified CMH test. Treatment comparisons in the prospective menstrual trial were performed using a CMH test (one-sided *P* value).

Abbreviations: BTK, behind-the-knee; CMH, Cochran–Mantel–Haenszel; SE, standard error.

Source: From Ref. 50.

twice daily with a standard scoring scale (69). The protocol allows for testing under different conditions, for example, testing on hydrated skin (by pretreating the application sites with a wet bandage under occlusion) or on compromised skin (e.g., by tape stripping the SC or by pretreating the application sites with mild surfactant). See chapter 51 of this book.

Modified Skin Patch Tests for Chemical Irritation

Patch testing on the back or upper arm has been used historically to evaluate potential chemical irritation from raw materials and product formulations. Modifying the conditions of the traditional patch test to increase sensitivity to mild substances has proved difficult. We examined four variations of the traditional, four-day, semiocclusive patch test, that is, combinations of either wet or dry test materials applied to either intact or compromised skin (65). When commercial sanitary pads were tested in this manner, none of the protocol modifications increased test sensitivity. Moreover, none of the protocol modifications revealed significant differences in paired comparisons of products. In short, no meaningful increase in sensitivity was achieved for assessing materials with inherently low irritation potential.

As an alternative approach to increasing test sensitivity, we attempted to identify subpopulations that may be inherently more sensitive to irritant effects. A large percentage of people in Western industrialized countries (30–70%) consider their skin to be sensitive (13,70,71); moreover, in a U.S. survey of 1039 adults, 57% of Caucasian and 66.7% of African-American women perceived their genital skin to be sensitive (13,72). One caveat to defining the test population is that "sensitive skin" is a self-declared condition lacking objective diagnostic criteria. A significant fraction of people who declare their skin to be sensitive show no increase in objective responses to chemical probes, whereas individuals who do not believe their skin to be sensitive sometimes respond strongly (73). Consequently, the challenge is to identify an appropriate susceptible population for testing. A preliminary study in our laboratory screened 222 subjects to find a group with a self-reported history of reactions to personal products or clothing and other dermatologic complaints who also considered their skin to be sensitive. Of those screened, 83 (37%) reported a history of reactions to products and clothing in conjunction with a general history of dermatologic complaints, but of those, only 15 (7% of the total respondents) declared their skin to be sensitive most or all of the time (68). In a standard four-day patch test, these subjects displayed directionally higher erythema scores to physiologic saline (nonirritant control), dilute SLS (irritant control), as well as two different sanitary pads than has been observed historically with these materials, an observation that suggests the subgroup was more responsive to topically applied substances (68).

Alternatively, some evidence exists that women who report facial skin sensitivity exhibit a higher level of objectively assessed vulvar irritation. In a prospective trial of sanitary pads, subgroups of women who presented with clinically observable vulvar erythema at study entry were more likely to report facial redness, although they did not necessarily report their genital skin to be sensitive (74). This may reflect the fact that changes in facial redness are more obvious to the casual observer. More research is needed to assess whether facial sensitivity may be a surrogate marker for sensitive vulvar skin.

Sensitive Techniques for Detecting Inflammatory Changes

Enhanced Visualization with Cross-Polarized Light

Conventional visual scoring for erythema and skin dryness has been the mainstay for detecting the inflammatory effects of topical products. Visual scoring is reliable and reproducible when performed routinely by trained graders (75). However, the ability to detect subclinical alterations—before inflammation becomes clearly visible—will allow materials with low irritancy potential to be discriminated more readily (76). Moreover, detecting subclinical inflammation might aid in understanding the currently elusive connection between irritancy and the syndrome of sensitive skin.

One promising technique is illumination with cross-polarized light (Syris v600™ Visualization System, Syris Scientific LLC, Gray, ME, USA), which enables subsurface visualization of the tissue and vasculature to a depth of about 1 mm (51). Visualization with cross-polarized light was more effective at detecting mild irritation produced by a low concentration of a standard irritant (0.01% SLS) in a standard four-day patch test (52). It also enabled differentiation of the irritation potential of two very similar sanitary pads after only a single application in the BTK clinical test (52). Moreover, as discussed in the Evaluation of Subjective Sensory Effects section of this chapter, subclinical inflammation visualized with this technique might correlate with sensory effects that occur in the absence of visually apparent surface inflammation.

Measurement of Molecular Markers of Inflammation

Another sensitive detection technique under investigation is the measurement of molecular markers of inflammation, such as the cytokines IL-1, IL-1RA, and IL-8. With this noninvasive technique, suitable for use on infants or adults, the cytokines are absorbed on tape applied to compromised or inflamed skin, then extracted with saline and analyzed by immunoassay. Adult skin treated under patch with a standard irritant (SLS) exhibited significantly higher levels of IL-1 in the absence of visible irritation than untreated skin. Higher levels of this cytokine were also recovered from areas of erythema, heat rash, and diaper rash on diapered infant skin compared with clinically normal skin on the upper thighs of infants (53).

Evaluation of Subjective Sensory Effects

Analyzing the frequency and severity of reported sensory effects from either predictive tests or from prospective product trials might increase the ability to detect subtle differences in tolerability or consumer preference. Simple questionnaires on unpleasant sensations, such as burning, itching, pain, chaffing, or dryness, are administered routinely in prospective clinical trials of sanitary pads (77) and feminine wipes (78). Such questionnaires have been incorporated into the BTK clinical test (53).

The most intriguing insights have been obtained by correlating sensory effects reported in the BTK test protocol with concurrent subclinical inflammation visualized with cross-polarized light. We tested two sanitary pads (designated A and B) that appeared to differ in tolerability based on in-market consumer surveillance but were not distinguished by objective skin grading in standard skin patch tests (54). Unaided visual scoring in the

BTK test discriminated the irritancy potential of the two products after the third day of a four-day exposure; however, when subsurface visualization with cross-polarized light was used, the two products were discriminated at every evaluation point beginning with the first exposure ($P < 0.05$) (54).

Differences in subsurface inflammation between the tested products also correlated with the relative frequency of reports of a burning sensation experienced during the study. The relative frequency of reports of a burning sensation at each evaluation point in the BTK clinical test discriminated the in-market tolerability of two products with an even higher level of confidence ($P < 0.001$) than did the level of subsurface inflammation. Hence, the analysis of subjective reports of unpleasant sensory effects in a sensitive protocol, such as the BTK clinical test, when corroborated by concurrent subclinical inflammation, could provide a new level of discriminating power to predict subtle differences in topical effects on specialized epithelia, such as the vulva.

Interestingly, we found that observing subsurface inflammation concurrently on the introitus, vagina, and cervix of women with a normal vulva distinguished patients with vestibulodynia (vulvar vestibulitis syndrome) from healthy patients and patients with other types of vulvovaginitis (52). Vestibulodynia, considered a sensory disorder, is a perplexing condition marked by provoked vestibular pain without clinical evidence of inflammation.

CONCLUSIONS

This review contrasts the characteristics of exposed skin and vulvar tissue, presenting a rationale for the heightened vulvar susceptibility to topical agents. Factors that may contribute to this heightened susceptibility include the elevated blood flow, skin hydration, and friction coefficient of vulvar skin; reductions in epithelial thickness and keratinization in moving from the labia majora to the labia minora and vulvar vestibule; and the heightened permeability of the nonkeratinized mucosa.

Although the potential for elevated vulvar susceptibility to topical agents is not conclusively established, the clinical evidence is suggestive. Epidemiologic studies indicate that the contribution of topical medications and personal products to vulvar allergic contact dermatitis is substantial (20) and a variety of substances have been implicated in contact sensitization from vulvar exposure (19).

The safety assessment of products that contact the vulva must account for its potentially heightened susceptibility to topical agents. Clinical patch tests on exposed skin, part of the standard repertoire for premarket assessment of topical products, may not sufficiently mimic the characteristics of vulvar exposure. To adapt the safety assessment arsenal for such specialized epithelia, three approaches have been implemented: (*i*) cutaneous test methods have been modified to increase their sensitivity for detecting topical effects, (*ii*) more sensitive methods of discerning epithelial inflammation have been incorporated into predictive testing, and (*iii*) greater conservatism is being employed in the risk assessment process when extrapolating from the skin to vulvar exposures.

Test protocols that may be employed in the safety assessment of topical effects on the vulva include the following: (*i*) the BTK clinical test, which assesses chemical and frictional irritation induced by repeated application to the popliteal fossa and has been shown to predict comparative irritation from product use; (*ii*) the mFCAT, which assesses chemical and frictional effects from repeated wiping across the epithelial surface and has distinguished the irritation potential of mild articles; and (*iii*) a modified HRIPT protocol, which increases the induction phase of exposure by 67%, was proposed to assess materials intended for vulvar contact and should heighten test sensitivity based on theoretic considerations.

As adjunct to these protocols, sensitive techniques for detecting subclinical inflammation (such as subsurface visualization with cross-polarized light and analysis of cytokines mediators of inflammation) have been incorporated into predictive testing to lower the detection limits for inflammation. Moreover, the analysis of subjective sensory effects reported in predictive cutaneous testing, coupled with concurrent detection of subclinical inflammation, shows promise for discriminating subtle differences in the tolerability of products that contact the vulva. These combined approaches augment the sensitivity of predictive testing, and when used judiciously in the risk assessment process, support an appropriate level of conservatism in the safety evaluation of materials that contact the vulva.

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55 Biomarkers associated with severe cutaneous adverse reactions

Nahoko Kaniwa and Yoshiro Saito

INTRODUCTION

Although skin rash is a frequently experienced adverse drug reaction, Stevens–Johnson syndrome (SJS), toxic epidermal necrosis (TEN), and drug-induced hypersensitivity syndrome (DIHS) are life-threatening severe cutaneous adverse reactions accompanied by fever and systemic complications. SJS and TEN, with characteristic mucosal disorders, are considered to be variations of the same disease expressed with different levels of severity (1), although this designation is controversial. The most widely accepted classification is based on the level of skin detachment area as follows; a skin detachment level less than 10% of the body surface, SJS; a skin detachment level from 10% to less than 30% of the body surface, SJS-TEN overlap; and a skin detachment level not less than 30%, TEN (1), while in Japan TEN is defined as a severity of skin detachment more than 10% of the body surface (2). DIHS, also called DRESS or HSS as acronyms for drug reaction with eosinophilia and systemic symptoms or hypersensitivity syndrome (3,4), is a severe disease with multiorgan failure and has been proposed to associate with reactivation of herpes virus-6 (5).

SJS/TEN and DIHS are idiosyncratic adverse reactions and the events are not usually dependent on the dose or plasma level of the causative drug. Moreover, these diseases are generally not identified during the drug development process due to their extremely low incidence but are initially recognized after the broad use of a drug in the post-approval period. These factors complicate the ability of physicians to administer drugs safely. Recently, several types of human leukocyte antigens (HLAs) have been reported to be associated with particular drug-induced severe cutaneous adverse reactions, which establish them as promising predictors for such reactions.

HLA AND OTHER GENOMIC BIOMARKERS RELATED TO SEVERE CUTANEOUS ADVERSE REACTIONS

HLAs are proteins involved in immune reactions. HLA-A, HLA-B, and HLA-C are categorized as class I molecules, which are ubiquitously expressed on the surface of cells including keratinocytes; and HLA-DR, HLA-DQ, and HLA-DP are categorized as class II molecules, which are mainly expressed on the surface of antigen presenting cells such as B-cells, macrophages or dendritic cells. Coding genes of all HLAs are on the short arm of chromosome 6 and are known to be diversely polymorphic. For example, more than 1000 alleles of *HLA-A*, *HLA-B*, and *HLA-C* have been identified to date (6).

Carbamazepine-Induced Severe Cutaneous Adverse Reactions

Chung et al. found for the first time a strong association between carbamazepine-induced SJS/TEN and *HLA-B*1502* in Han Chinese living in Taiwan (7). They reported that the carrier frequency of *HLA-B*1502* in these cases (44/44, 100%) was significantly higher than in carbamazepine-tolerant patients (3/101, 3%) ($p = 3.13E-27$), and that the odds ratio was 2504 (95% confidence interval (CI); 126–49,522). As shown in Table 55.1, this strong association has been confirmed by their continuing follow-up study (8), and studies involving Han Chinese patients in Hong Kong (9), Asian-originating patients living in Europe (10), Indian patients (11), and Thai patients (12–14). *HLA-B*1502* was also found to be a risk factor for carbamazepine-induced SJS/TEN in mainland Chinese similar to its being a risk factor for Southeastern Asian patients (15,16). However, the association of carbamazepine-induced SJS/TEN with *HLA-B*1502* has not been found in European (10), Japanese (17,18), or Korean patients (19). Moreover, no associations of *HLA-B*1502* with other carbamazepine-induced cutaneous adverse reactions such as maculopapular exanthema (MPE) or HSS were identified even with Thai patients (10) or Han Chinese patients in Taiwan (8) in addition to there being no correlations with Japanese (20), Korean (19), or European patients (21).

The incidence of carbamazepine-induced SJS/TEN in some Southeastern Asian countries such as Taiwan, Thailand, Malaysia, and the Philippines is 10-fold higher than in European countries, the United States, and Japan (22,23). Population allele frequencies of *HLA-B*1502* in Southeastern Asian countries are much higher (2–12%) than in Caucasians (rare) and in East Asian countries (Japan and Korea, 0–0.4%) (24), and this HLA type may be causative for the higher incidence of carbamazepine-induced SJS/TEN observed in Southeastern Asian countries.

Although carriers of *HLA-B*1502* have not been detected, Kaniwa et al. found four carriers of *HLA-B*1511* from 14 Japanese carbamazepine-induced SJS/TEN patients (18). *HLA-B*1511* and *HLA-B*1502* belong to the same serotype HLA-B75. Other major members of HLA-B75 are *HLA-B*1508* and *HLA-B*1521*. To date carbamazepine-induced SJS/TEN patients in Asia who carry *HLA-B*1511* (13,18,19), *HLA-B*1508* (11), and *HLA-B*1521* (13) have been reported, and particularly in East Asian countries such as Japan (18) and Korea (19), allele frequencies of *HLA-B*1511* in carbamazepine-induced SJS patients are much higher than carbamazepine-tolerant or healthy control subjects (Table 55.1). Although the alleles *HLA-B*1508*,

TABLE 55.1
Associations Between HLA Alleles and Carbamazepine-Induced Severe Cutaneous Adverse Reactions

Biomarker (HLA Allele)	Disease Phenotype	Ethnic Group	Carrier Frequency in Cases	Allele Frequency in Cases	Carrier Frequency in Controls	Allele Frequency in Controls	p-value	Odds Ratio (95% CI)	Reference
*B*1502*	SJS/TEN	Han Chinese in Taiwan	59/60		6/144		2.6E-41	1357 (193.4–8838.3)	(8)
		Asians living in Europe	4/4						(10)
		Han Chinese in Hong Kong	4/4						(9)
		Indians	6/8		0/10		0.0014	71.40 (3.0–1698)	(11)
		Thai	6/6		8/42		0.0005	25.5 (2.68–242.61)	(12)
		Thai	37/42		5/42		2.89E-12	54.76 (14.62–205.13)	(13)
		Han Chinese in mainland	9/9		11/80		<0.001	114.8 (6.3–2111.0)	(15)
		Chinese in mainland	16/17		2/21		<0.0001	152 (12–1835)	(16)
		Europeans	0/8						(10)
		Japanese	0/14						(18)
		Koreans	1/7		1/50		N.S.		(19)
	Hypersensitivity	Caucasians	0/56		0/43				(20)
	MPE/HSS	Han Chinese in Taiwan	1/31		6/144		N.S.		(8)
	MPE	Thai	2/9		8/42		N.S.		(12)
	MPE/HSS	Han Chinese in mainland	10/39		11/80		N.S.		(15)
*B*1508*	SJS/TEN	Indians	1/8						(11)
*B*1511*	SJS/TEN	Thai	1/42						(13)
		Japanese	4/14	4/28		10/986[b]	0.0004[c]	16.3 (4.76–55.6)	(18)
		Koreans	3/7		250		0.011	18.0 (2.3–141.2)	(19)
*B*1521*	SJS/TEN	Thai	2/42						(13)
*A*3101*	SJS	Europeans	5/12		10/257		8.0E-5	25.93 (4.93–116.18)	(26)
	SJS/TEN	Japanese	5/6		54/420		2.35E-4	33.9 (3.9–295.6)	(28)
	SCARs[a]	Japanese		11/44		53/742[b]	0.0004[c]	4.33 (2.07–9.06)	(27)
	SCARs[a]	Koreans	13/24		7/50		0.001	7.3 (2.3-22.5)	(19)
	MPE/HSS	Han Chinese in Taiwan	8/31		4/144		0.0021	12.17 (3.6–41.2)	(8)
	DIHS	Japanese	21/36		54/420		2.06E-9	9.5 (4.6–19.5)	(28)
	Others than SJS/TEN/DIES	Japanese	19/35		54/420		4.74E-8	8.0 (3.9–16.6)	(28)
	HSS	Europeans	10/27		10/257		0.03	12.41 (1.27–121.0)	(26)
	MPE	Europeans	23/106		10/257		8.0E-7	8.33 (3.59–19.36)	(26)

[a]Severe cutaneous adverse reactions.
[b]Healthy control.
[c]Allele frequencies between cases and controls were compared.
Abbreviations: DIHS, drug-induced hypersensitivity syndrome; HSS, hypersensitivity syndrome; MPE, maculopapular exanthema; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrosis.

*HLA-B*1511*, and *HLA-B*1521* are not as common as the allele *HLA-B*1502* in Southeastern Asians, the allele frequencies in Asians (0–3%) are higher than those in Caucasians (24). A recent *in vitro* study suggested the potential involvement of HLA-B*1511, HLA-B*1508, and HLA-B*1521 expressed in KERTrs in binding to CBZ and CTL activation (25). Thus, some members of the HLA-B75 serotype as well as *HLA-B*1502* are also risk factors for the development of carbamazepine-induced SJS/TEN in Asian countries.

In addition to *HLA-B*1502* or *HLA-B75* serotype, *HLA-A*3101* which was previously reported as a risk factor for carbamazepine-induced HSS and MPE in Han Chinese (8) has been reported to be a biomarker for carbamazepine-induced cutaneous adverse reactions in Europeans (26), Japanese (27,28), and Koreans (19) as shown in Table 55.1. In these three populations, *HLA-A*3101* is a risk factor for various carbamazepine-induced cutaneous adverse reactions ranging from mild skin rash such as MPE to sever cutaneous adverse reactions including SJS/TEN, while it is a risk factor for MPE and HSS but not for SJS/TEN in Taiwanese (8). Genome-wide association studies have detected SNPs (single nucleotide polymorphisms) on chromosome 6 close to the region of *HLA-A* that are in strong linkage disequilibrium with *HLA-A*3101* (26,28).

Thus, genetic biomarkers for carbamazepine-induced cutaneous adverse reactions are ethnic specific as well as probably phenotype specific.

Other Aromatic Antiepileptics-Induced Severe Cutaneous Adverse Reactions

Other aromatic antiepileptics such as phenytoin, lamotrigine, and phenobarbital also often cause cutaneous adverse reactions. Biomarkers related to these aromatic antiepileptics-induced cutaneous adverse reactions are summarized in Table 55.2, although the associations are typically weaker or less well established than the carbamazepine cases.

TABLE 55.2
Associations Between HLA Alleles and Aromatic Anti-Epileptics-Induced Severe Cutaneous Adverse Reactions

Causative Drug	Biomarker (HLA Allele)	Disease Phenotype	Ethnic Group	Carrier Frequency in Cases	Allele Frequency in Cases	Carrier Frequency in Controls	Allele Frequency in Controls	P-value	Odds Ratio (95% CI)	Reference
Phenytoin	*B*1502*	SJS/TEN	Taiwanese and Chinese	8/26		9/113		0.0041	5.1 (1.8–15.1)	(29)
		SJS/TEN	Thai	4/4		8/45		0.005	15.8 (1.82–188.4)	(12)
		MPE	Thai	3/4		8/45		N.S.		(12)
	*B*1301*	SJS/TEN	Taiwanese and Chinese	9/26		14/113		0.0154	3.7 (1.4–10.0)	(29)
Lamotrigine	*B*1502*	SJS/TEN	Taiwanese and Chinese	2/6						(29)
		TEN	Han Chinese in Hong Kong	1/1						(9)
		SJS/TEN	Han Chinese	1/3		1/21		N.S.		(32)
		MPE	Han Chinese	2/22		1/21		N.S.		(32)
		SJS/TEN	Han Chinese		0/4		4/56	N.S.		(33)
		MPE	Han Chinese		1/22		4/56	N.S.		(33)
	*B*5801*	SJS/TEN HSS	Mainly Europeans		3/44		0/86	0.037		(34)
Phenytoin, phenobarbital, or carbamazepine										
	*B*1301*	DIHS	Japanese	4/13						(20)
Oxycarbazepine										
	*B*1502*	SJS/TEN	Taiwanese and Chinese	3/3						(29)

Abbreviations: DIHS, drug-induced hypersensitivity syndrome; HSS, hypersensitivity syndrome; MPE, maculopapular exanthema; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrosis.

For phenytoin-induced Han Chinese SJS/TEN patients of Taiwan, Hung et al. reported that the carrier frequency of *HLA-B*1502* (8/26, 30.8%) was significantly higher than for tolerant controls (9/113, 8.0%), with an odds ratio of 5.1 (95% CI; 1.8–15.1, p = 0.0041) (29). For patients from Thailand, all of the four patients with phenytoin-induced SJS/TEN carried *HLA-B*1502*, and the association was significant in comparison to the carrier frequency of phenytoin-tolerant patients (1/9), while no associations were observed between *HLA-B*1502* and phenytoin-induced MPE (12). Man et al. detected a phenytoin-induced SJS/TEN case with *HLA-B*1502* and five phenytoin-induced MPE cases without *HLA-B*1502* (9). There are two case reports from mainland China on phenytoin-induced SJS/TEN patients; one report of two patients with the negative *HLA-B*1502* allele (30) and another report of one patient out of two with a positive *HLA-B*1502* allele (31). These results indicate that *HLA-B*1502* seems to be at least one of the risk factors for development of SJS/TEN caused by phenytoin in Southeastern Asian countries, although the association is not as strong as in the case of carbamazepine-induced SJS/TEN. Hung et al. also found a moderate association between *HLA-B*1301* and phenytoin-induced SJS/TEN (29).

For lamotrigine-induced SJS/TEN, two patients out of six from Taiwan (29) and one patient from Hong-Kong (9) carried *HLA-B*1502*. However, two recently performed case control studies using Han Chinese lamotrigine-induced patients including SJS/TEN and MPE failed to show an association with *HLA-B*1502*. A weak association of lamotrigine-induced severe cutaneous adverse reactions with *HLA-B*5801*, a known risk factor for allopurinol-induced severe cutaneous adverse reactions, discussed later, was detected in Europeans (34). It is interesting that two out of six Chinese lamotrigine-induced SJS patients also carried *HLA-B*5801* (29). Further large scale studies are required to elucidate genetic associations with lamotrigine-induced severe cutaneous adverse reactions.

Kano et al. pointed out a possible association between *HLA-B*1301* and a particular virus reactivation based on the observation that three out of four Japanese patients carrying *HLA-B*1301* had cytomegalovirus reactivation during the course of DIHS/DRESS caused by phenytoin, carbamazepine or Phenobarbital (20).

Allopurinol-Induced Severe Cutaneous Adverse Reactions

Allopurinol, one of the most often used drugs for hyperuricemia, is the most common cause of SJS/TEN in Europe (35). For the first time, a strong association between allopurinol-induced severe cutaneous adverse reactions and *HLA-B*5801* for Han Chinese in Taiwan was found by Hung et al. (Table 55.3) (36). This association has been confirmed in patients for Thailand (37), Japan (38,17,39), Europe (40), and Korea (41), although the strength of the association is dependent on the ethnic groups. Interestingly, the biomarker *HLA-B*5801* is a predictor for a wide variety of cutaneous reactions including SJS/TEN, HSS, and MPE unlike in the case of *HLA-B*1502*, which is a risk factor only for SJS/TEN. *HLA-B*5801* is a rather common allele (population allele frequencies are greater than 5%) in ethnic groups in which high sensitivity was observed in case–control studies, while population allele frequencies of *HLA-B*5801* are very low in Japanese and Europeans (less than 1%) (24). SNPs on chromosome 6 are completely linked with *HLA-B*5801* as detected by a genome-wide association in Japanese (39), and as such, they could be used as surrogate biomarkers in screening tests for *HLA-B*5801* prior to the initiation of allopurinol therapy.

TABLE 55.3
Associations Between HLA Alleles and Severe Cutaneous Adverse Reactions Caused by Various Drugs

Causative Drug	Biomarker (HLA Allele)	Disease Phenotype	Ethnic Group	Carrier Frequency in Cases	Allele Frequency in Cases	Carrier Frequency in Controls	Allele Frequency in Controls	P-value	Odds Ratio (95% CI)	Reference
Allopurinol	*B*5801*	SJS/TEN/HSS	Han Chinese in Taiwan	51/51		20/135		4.7E-24	580.3 (34.4–9780.9)	(36)
		SJS/TEN	Thai	27/27		7/54		1.61E-13	348.3 (19.2–6336.9)	(37)
		SJS/TEN/DIHS	Japanese	3/3						(38)
		SJS/TEN	Japanese		10/36		6/986[a]	5.39E-12[b]	62.8 (21.2–185.8)	(39)
		SJS/TEN	Europeans	15/27		28/1822[a]		<E-8	80	(40)
		SJS/TEN/DIHS	Koreans	24/26		6/57		2.45E-11	97.8 (18.3–521.5)	(41)
Abacavir	*B*5701*	HSS	Western Australian	14/18		4/167		<0.0001	117 (29–481)	(42)
	*B*5701 _HLA-DR7_ HLA-DQ3*	HSS	Western Australian	13/18		0/167			822 (43–15,675)	(42)
	*B*5701*	HSS	Western Australian	17/18		4/230		<0.0001	960	(43)
	*B*5701_ Hsp70-Hom M493T*	HSS	Western Australian	17/18		1/230		<0.00001	3893	(43)
	*B*5701*	HSS	Caucasians	Not indicated		Not indicated		8.4E-23	21.4 (9.5–48.1)	(45)
	*B*5701*	HSS	Hispanics	Not indicated		Not indicated		2.1E-4	30.4 (1.74–530.9)	(45)
	*B*5701*	HSS	Africans	Not indicated		Not indicated		0.27		(45)
	*B*5701*	HSS	Caucasians	57/129		8/202			19 (8–48)	(46)
	*B*5701*	HSS	Caucasians (cases with positive skin patch test result)	42/42		8/202			1945 110–34,352	(46)
	*B*5701*	HSS	Africans	10/69		2/206			17 (4–164)	(46)
	*B*5701*	HSS	Africans (cases with positive skin patch test result)	5/5		2/206			900 (38–21,045)	(46)
Methazolamide	*B59*	SJS	Japanese	3/3						(53)
		SJS	Koreans	6/6						(55)
		SJS/TEN	Koreans	2/2						(56)
	*B*5901*	SJS/TEN	Koreans	5/5		20/485[a]		<0.001	249.8 (13.4–4813.5)	(57)
Acetazolamide	*B59*	SJS	Koreans	2/2						(58,59)
Nevirapine	*B*3505*	Skin rash	Thai	25/143		2/181		4.9E-8	18.96 (4.87–73.44)	(60)

[a]Healthy control.
[b]Allele frequencies between cases and controls were compared.
Abbreviations: DIHS, drug-induced hypersensitivity syndrome; HSS, hypersensitivity syndrome; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrosis.

Abacavir-Induced Hypersensitivity Syndrome

Abacavir is a commonly used nucleotide analog with antiviral activity against HIV-1. Approximately 5–9% of patients develop HSS within six weeks after initial exposure to abacavir (42). Mallal et al. first reported the association between abacavir-induced HSS and *HLA-B*5701* in Western Australian HIV patients as shown in Table 55.3 (42). In Western Australian patients groups, *HLA-B*5701* is highly linked with *HLA-DR7* and *HLA-DQ3* (serotypes of HLA class II molecules) (42), or with a nonsynonymous SNP, *Hsp70-Hom M493T* (Heat shock 70 kDa protein 1L) (43), and the odds ratios for combinations of these genomic markers (822 for *B*5701_HLA-DR7_HLA-DQ3*, and 3893 for *B*5701_ Hsp70-Hom M493T*) were much higher than the odds ratio (100) with *HLA-B*5701* alone (42). Initially, an association between *HLA-B*5701* and abacavir-induced HSS was ambiguous in African patients (44,45). Hughes et al. examined the association between *HLA-B*5701* and abacavir-induced HSS in various ethnic groups with a cohort study in which patients from 12 countries participated. They confirmed an association between *HLA-B*5701*

and abacavir-induced HSS in Caucasian males and females and Hispanic, but failed to detect a significant association in African (45). As shown in Table 55.3, the carrier frequency of *HLA-B*5701* in abacavir-induced HSS in African patients seemed to be low (14%) compared with similar Caucasian patients (44%) in a study performed in the United States by Saag et al. (46) However, for patients immunologically confirmed by skin patch test using 1% and 10% abacavir solutions, the carrier frequencies of *HLA-B*5701* were 100% both in Caucasian (42/42) and African patients (5/5) (46). Thus, *HLA-B*5701* is now regarded as being a risk factor for abacavir-induced HSS both for Caucasians and Black people.

*HLA-B*5701* is a common allele in Caucasians that has recently been found to also be a risk factor for flucloxacillin-induced liver injury (47). However, *HLA-B*5701* is a rare allele in Asian countries such as Taiwan (48), Japan (49), and Korea (50). Only one out of 320 patients with HIV carried *HLA-B*5701* in Taiwan (48), and no carriers were found in 534 Korean patients with HIV (50). The availability of a screening test for *HLA-B*5701* did not affect the incidence of abacavir-induced HSS in Koreans (screening unavailable (3/57) versus screening available (4/93)) (50). Abacavir-induced HSS is encountered less frequently in Japan (1.3%) (51) and Taiwan (0.9%) (48) compared with Western countries.

Methazolamide-Induced SJS/TEN

Methazolamide, a sulfonamide derivative, is a carbonic anhydrase inhibitors used to lower the intraocular pressure in glaucoma. Most reports on methazolamide-induced SJS/TEN have been related to Japanese (52,53) and Koreans patients (54–56), and some of these reports (53,36,37) have suggested a strong association between *HLA-B59* serotype and SJS/TEN (Table 55.3). A recent case–control study performed in Korea using five cases and 485 control subjects from the general population revealed that *HLA-B*5901* is a risk factor for methazolamide-induced SJS/TEN (57). Case reports (58,59) on Korean acetazolamide-induced SJS patients positive for *HLA-B59* suggest that *HLA-B59* (*HLA-B*5901)* is also a risk factor for SJS/TEN caused by acetazolamide, another sulfonamide derivative carbonic anhydrase inhibitor. The allele frequency of *HLA-B*5901* is 1–2% in Japanese and Korean, but is very rare in Caucasians and Blacks (24).

Nevirapine-Induced Cutaneous Adverse Reactions

Nevirapine is a potent non-nucleoside reverse transcriptase inhibitor used for the treatment of HIV-1 infection and is known to often cause various types of skin rash. The *HLA-B*3505* allele was observed for 17.5% of Thai patients with nevirapine-induced skin rash compared with only 1.1% of nevirapine-tolerant Thai patients (OR =18.96; 95% CI: 4.87–73.44, $Pc = 4.6 \times 10^{-6}$) and 0.7% of the general Thai population (OR = 29.87; 95% CI = 5.04–175.86, $Pc = 2.6 \times 10^{-5}$) (60). Thus, *HLA-B*3505* allele is a strong predictor for nevirapine-induced skin adverse reactions in HIV-infected Thai patients. A genome-wide association study identified variations in *CCHCR1* associated with *HLA-B*3505* (61), which could be used as surrogate predictors for screening patients prior to the initiation of nevirapine treatment.

SJS/TEN with Ophthalmic Sequelae

Mucocutaneous damage caused by SJS/TEN often inflicts severe, lifelong visual dysfunction. The carrier frequency of *HLA-A*0206* in Japanese patients with visual dysfunction after SJS/TEN (30/71, 42.3%) was significantly higher than in Japanese healthy volunteers (17/113, 15.0%) (62,63). In addition, the Toll-like receptor 3 gene (TLR3) polymorphisms, rs293248 and rs299698, have been suggested to be associated with ocular surface complications in Japanese SJS/TEN patients (64).

Genome-Wide Association Studies

Currently high-throughput technologies such as genome-wide SNP analysis are available for exploring biomarkers associated with severe adverse reactions; for example, biomarkers related to drug-induced liver injury caused by flucloxacillin (47) and myopathy caused by simvastatin (65) have been successfully detected by genome-wide association studies. For severe cutaneous adverse reactions, SNPs strongly linked with known biomarkers such as *HLA-B*5801* (39), *HLA-A*3101* (26,28) and *HLA-B*3505* (61) have been additionally found by GWAS of allopurinol-, carbamazepine- and nevirapine-induced severe cutaneous adverse reactions, respectively, as mentioned earlier. However, two recently performed genome-wide association studies on skin rash with large sample sizes and multiple causative drugs that include 424 European cases and 1881 control subjects (66), and 96 cases and more than 4000 control subjects (67), failed to detect new biomarkers, although SNPs associated with *HLA-B*5801* were detected by the former study performed by the RegiScar group. This result was presumably due to the involvement of a considerable number of patients with allopurinol-induced skin rash. As we have mentioned, biomarkers for severe cutaneous adverse reactions are usually causative drug-specific and have generally been identified by case–control studies on each causative drug with the exception of studies on patients with complicating visual dysfunction (62–64). Therefore, if a genome-wide association study with a large sample size is applied to a particular causative drug-induced cutaneous adverse reaction, new genomic biomarkers other than *HLA*-related genes might be found.

OTHER BIOMARKERS

Several biomarkers related to the pathophysiology of SJS/TEN have been reported. Epidermal skin detachment is a characteristic of SJS/TEN, and several cytotoxic proteins released from immune cells are known to be involved in epidermal keratinocyte apoptosis/necrolysis including Fas–Fas ligand (FasL) interaction and soluble cytotoxic factors such as granulysin (68).

The soluble form of FasL (sFasL) was reported to be released from peripheral blood mononuclear cells (PBMCs) by stimulation of causal drugs and to induce keratinocyte apoptosis in SJS/TEN patients (69). Furthermore, in a pilot study using four previously identified SJS/TEN patients induced by carbamazepine, their PBMC secreted approximately twice the amount of sFasL as in two healthy, age-matched control subjects, and sFasL release increased in a culprit drug concentration-dependent manner (70). Thus, sFasL was thought to be a potential biomarker for SJS/TEN. Indeed, two to four days before the onset of SJS/TEN, serum sFasL levels increased to more than 100 pg/mL in five out of seven patients, where the disease onset was defined as erosion/ulceration of mucocutaneous regions or first development of ocular lesion (71). These increases rapidly diminished within five days of disease onset, and were not observed throughout the same time period in 33 patients with ordinary types of drug-induced skin reactions as well as in 32 healthy control subjects. Thus, serum

TABLE 55.4

Performance Characteristics of Screening for *HLA-B*5701* in the Control Group

	HLA-B*5701			Performance Characteristics[a]
	Positive	Negative	Total	
Clinical diagnosis				
HSS	30	36	66	Sensitivity 45.5%
No reaction	19	762	781	Specificity 97.6%
				PPV 61.2%, NPV 96.5%
Immunologically diagnosis				
HSS	23	0	23	Sensitivity 100%
No reaction	25	794	819	Specificity 96.9%
				PPV 47.9%, NPV 100%

Abbreviations: HSS, hypersensitivity syndrome; NPV, negative predictive value; PPV, positive predictive value.

sFasL levels could be a predictive biomarker for the onset of SJS/TEN. However, serum levels of sFasL were too low to use in a rapid diagnostic device.

Blister cells from skin lesions of SJS/TEN predominantly consist of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (72). These blister fluids were cytotoxic against keratinocytes and were found to contain cytotoxic proteins including granulysin, which were released from CTLs and NK cells. The cationic protein granulysin was detected at ~7 μg/mL in the blister fluids, which was two to four orders of magnitude higher than other cytotoxic proteins, perforin (~30 ng/mL), granzyme B (~5 ng/mL), and sFasL (~0.4 ng/mL) in the blister fluids from the afflicted skin regions of the SJS/TEN patients. Furthermore, the increase in granulysin levels was specific to SJS/TEN and depending on the severity (the levels were TEN > overlapping SJS-TEN>SJS), and not found in similar skin lesions from burn injury or bullous pemphigoid. Thus, the skin detachments in SJS/TEN could be attributed primarily to granulysin. Granulysin is also detected in the sera of SJS/TEN patients on the order of ng/mL from two to four days before onset of the disease (day 1 was defined the same as the above sFasL case) (73). If the cut-off value was set at 10 ng/mL (100-fold higher than sFasL level), four out of the five SJS/TEN patients were positive, but only one of 24 ordinary types of drug-induced skin reaction patients and none of the 31 healthy control subjects were positive. Serum granulysin levels decreased rapidly within five days after disease onset. Although this study used a small sample size and the results need to be confirmed, granulysin appears to be a promising biomarker for predicting the onset of SJS/TEN.

The levels of other biomarkers including serum lactate dehydrogenase and serum creatine kinase were also increased in SJS/TEN patients, but they are non-specific for the disease and only increase after the disease onset.

Not a biomarker, but the prognostic score for fatality is SCORTEN, a severity of illness score for TEN, which is now also applying to SJS (74). The SCORTEN evaluates seven independent factors within 24 h of admission: age ≥40 years old, malignancy, initial percentage of epidermal detachment ≥10%, tachycardia ≥120/min, serum urea >10 mM, serum glucose >14 mM, and serum bicarbonate <20 mM. These seven factors are allotted equal weighting in the score, and thus SCOTEN ranges from 0 (no factor present) to 7 (all factors present). Discriminating between death and recovery was shown to be excellent by several studies with a receiver operating characteristic (ROC) of approximately 80% (74–77). Recently, some alterations of the calculation methods have been introduced. The estimation three days after admission was more accurately predictive than the original first day calculation (78). In addition, an auxiliary method using only age, malignancy, and percentage of epidermal detachment ≥30% was proposed (76). This score does not depend on vital or laboratory parameters, and thus can easily apply to patients, but is poorer in discrimination by ROC curve analysis than the SCORTEN.

USEFULNESS OF GENOMIC BIOMARKERS FOR PREVENTION OF SEVERE CUTANEOUS ADVERSE REACTIONS AND CLINICAL IMPLEMENTATION

Biomarkers for severe cutaneous adverse reactions have largely been found through retrospective studies. Prospective randomized clinical studies could more efficiently evaluate the utility of screening for biomarkers to reduce drug-induced severe cutaneous reactions.

For this purpose, a prospective, randomized, multicenter, double-blind study called PREDICT-I was conducted to evaluate screening for *HLA-B*5701*, in which 1956 abacavir-naïve HIV-infected patients in Europe and Australia participated (79,80). In this study, patients were randomly divided into two groups; in one group, prospective screening for *HLA*5701* was applied to exclude *HLA-B*5701*-positive patients from abacavir treatment, while in the second group (control group), abacavir was given to all patients. The prevalence of *HLA-B*5701* in this study was 5.6%. The incidence of HSS in the group receiving prospective screening was significantly lower (3.4%) than in the control group (7.8%). Moreover, the screening completely eliminated HSS, immunologically confirmed with the epicutaneous patch test using abacavir solution in the prospective screening group (incidence 0%), while the incidence of immunologically confirmed HSS was 2.7% in the control group. The performance characteristics of *HLA-B*5701* screening in the control group were shown in Table 55.4. These results suggest that screening for *HLA-B*5701* prior to initiation of abacavir treatment can reduce the risk of hypersensitive reaction. Another large scale study performed using a racially diverse North American population (n = 725) also showed that prospective *HLA-B*5701* screening could reduce the risk of immunologically confirmed HSS to less than 1% among *HLA-B*5701*-negative individuals (81). A study conducted in France also indicated that prospective screening for *HLA-B*5701* reduced the incidence of suspected hypersensitivity from 22.5% to less than 1% and definite hypersensitivity from 12% to 0% (82). Moreover the rate of unwarranted interruption of abacavir therapy could be decreased from 10.2% to 0.73% possibly due to lowering the rate of false-positive diagnosis of hypersensitivity (82). Thus, all of these studies indicated the usefulness of prospective genetic-screening in lowering the incidence of abacavir-induced hypersensitivity. Currently, prospective screening for *HLA-B*5701* is required in Europe and the United States before the initiation of treatment with abacavir.

In Taiwan, a warning for *HLA-B*1502* as a risk factor for carbamazepine-induced SJS/TEN was introduced for the first time to the package inserts of carbamazepine products in December 2007, followed shortly by an announcement by the FDA. From 2002 to 2004, carbamazepine was newly prescribed to around 50,000 patients

each year, and the average incidence of carbamazepine-induced SJS/TEN during this period was estimated to be 0.22%, which corresponded to around 115 patients a year (83). A prospective study was performed in Taiwan in which 4877 subjects from 23 hospitals were recruited, and carbamazepine was administered to only *HLA-B*1502* negative patients (83). None of the 4120 patients who took carbamazepine and were followed for two months after the initiation of carbamazepine therapy developed SJS/TEN within two months. According to the above-mentioned historical data of the disease incidence, 10 patients would have been expected to develop SJS/TEN in this prospective study, and this difference was statistically significant ($p < 0.001$).

Prospective screening for *HLA-B*1502* and *HLA-B*5801* are now required in Taiwan before the initiation of therapy with carbamazepine and allopurinol, respectively. FDA has also approved a revision of product labels containing carbamazepine to clearly state that patients at genetically high risk should be screened for the *HLA-B*1502* allele before starting carbamazepine treatment.

CONCLUSIONS

Genomic biomarkers associated with the risk of severe cutaneous adverse reactions caused by several particular drugs have been accumulating, and some of these biomarkers are currently used in prospective screening before commencing drug therapy. However, some of the biomarkers are ethnic group-specific, and the strength of the association between biomarkers and adverse reactions is dependent on ethnic group. Even after excluding patients at high risk from treatment with a causative drug, clinical vigilance is still necessary for patients without relevant biomarkers during drug therapy. Biomarkers other than genomic risk factors could be useful for discriminating severe cutaneous adverse reactions from mild or moderate reactions, and they may be useful for determining the therapeutic management of patients.

Further *in vivo* and *in vitro* investigations are necessary to explore biomarkers for various drugs for which risk factors are unknown and to clarify the pathogenesis of severe cutaneous adverse reactions. In addition, the establishment of screening methods for use in the drug development process to identify drug candidates with a high risk for severe cutaneous reactions is also required.

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56 Decreasing allergic contact dermatitis frequency through dermatotoxicologic and epidemiologic-based interventions

Divya K. Alla, Naissan O. Wesley, and Howard I. Maibach

Allergic contact dermatitis (ACD) is an important occupational hazard, often leading to poor quality of life and significant financial loss for the workers it affects. Nickel allergy is one of the most common, and is highest among females and patients younger than 18 years, affecting 35.8% of patch-tested patients in North America. In contrast to Europe, where regulations have resulted in a decreasing prevalence of nickel allergy, the incidence of nickel ACD in North America is increasing (1). Denmark regulated the extent of nickel release in the ear-piercing process as well as nickel release from consumer products. In 1990 and 2006, 3881 18–69 year olds completed a postal questionnaire and were patch tested with nickel. The prevalence of concomitant nickel contact allergy and a history of hand eczema decreased among 18- to 35-year-old women from 9.0% in 1990 to 2.1% in 2006 ($P < 0.01$). Among older women, no significant changes were observed in the association between nickel contact allergy and hand eczema. This concludes that regulatory control of nickel exposure may have reduced the effect of nickel on hand eczema in the young female population (2). From 659 items covered by the EU Nickel Directive assessed with the dimethylglyoxime test, nickel release was shown for 9% of the tested items, all of which were intended for direct and prolonged contact with the skin. A high proportion of items bought at haberdashery shops and street markets, 34% and 61%, respectively, showed nickel release. It was suggested that authorities should monitor the market regularly and give attention to areas where compliance with the requirements is poor, for protection of public health (3). Observation by relating clinical epidemiologic data with recent chemical analyses of nickel release from costume jewellery between 1994 and 2009 indicate nickel allergy decreased in men (18–30 years) and in women (1–17 and 18–30 years); however, after 2000, there was no significant decrease in nickel allergy in the women aged 1–17 years. Exposure to nickel-containing products exceeding the (unnecessarily relaxed) permitted limit may explain why nickel contact allergy remains problem (4). According to part 2 of the EU Nickel Directive and the Danish nickel regulation, consumer items intended to be in direct and prolonged contact with the skin were not allowed to release more than 0.5 μg nickel/cm^2/wk. It was considered unlikely that nickel allergy would disappear altogether as a proportion of individuals reacted below the level defined by the EU Nickel Directive. Despite this, the EU Nickel Directive part 2 was expected to work as an operational limit that would sufficiently protect European consumers against nickel allergy and dermatitis. This review presents the accumulation of epidemiologic studies that evaluated the possible effect of this major public health intervention.

STUDIES

The Department of Occupational Dermatology, Madrid, Spain, conducted a study on 300 hairdressers seen from 1994 to 2003 and compared the results with those of a previous study of 379 hairdressers from 1980 to 1993. They found a significant increase in the frequency of positive patch-test responses (78.3 *vs* 58.8%) and OACD (58 *vs* 48.8%) with respect to the previous study. They also observed a significant increase in sensitization to most allergens, whereas a decrease was found in sensitization to thioglycolic acid (15.3–3%). The high frequency and increase of sensitizations among hairdressers highlights the need for indepth research and preventive intervention, such as avoiding the use of certain biocides in shampoo intended for professional use by hairdressers or further reducing the concentrations in oxidative hair dyes (5). Research from University of Erlangen–Nuremberg, Erlangen, Germany, indicated that for female patients who consulted centers of the Information Network of Departments of Dermatology between 01/2003 and 12/2006 the most important allergens were ammonium persulfate (21.7% positive), *p*-toluenediamine (19.6% positive), *p*-phenylenediamine (18.1% positive) and with decreasing time trend glyceryl monothioglycolate (2005/2006 still 7.5% positive) (6).

The results of patch testing performed from January 1998 to December 2006 at the Massachusetts General Hospital (MGH) have been analyzed and compared with our 1990–1997 data as well as with data from North American and European contact dermatitis societies. Data were collected from retrospective chart reviews and analyzed. The most common sensitizers were fragrance mix (18.3%) and nickel (16.7%). The results indicated that sensitization rates and the most important allergens at MGH have been stable over the past 17 years (7).

All topical pharmaceutic products marketed in Belgium, (that is, 3820 products), were examined as to their fragrance content as labeled. Data of 18,960 patients investigated for contact allergy between 1978 and 2008 were retrieved from our database, including information on the nature of the topical pharmaceutic products used, the results of patch tests, and the sensitization sources. Three hundred and seventy (10%) of 3280

of the topical pharmaceutical products were found to contain a total of 66 fragrance substances. Among 3378 patients suffering from iatrogenic ACD, 127 were found to react to 48 specific products, for which 38 different fragrance substances gave relevant positive reactions. Women were more affected than men, and legs, hands, and face were the most commonly affected body sites. This concludes that fragrances, the presence of which may be unnecessary, do contribute to iatrogenic ACD. Moreover, sensitized patients have difficulties in avoiding their specific allergens because standardized labeling of the ingredients in pharmaceutic products is lacking (8). In Europe, labeling of certain fragrance chemicals is mandated (9).

A pilot study has been done to assess the allergologic profile among the workers engaged in construction of roads and bridges in West Bengal, India. Sixteen workers were selected on clinical suspicion. Dermatitis affected exposed parts in 93.75% and covered areas in 62.5%. Total positive test was 24% and relevant 11%. The most common allergens were chromate [relevant allergy (RA): in 60% of patch tested workers], epoxy resin (RA: 30%), cobalt (RA: 20%), nickel (RA: 20%), thiuram mixture (RA: 10%), and black rubber mix (RA: 10%). Two cases (20%) had irritant contact dermatitis. The result indicated that chromate is the most frequent allergen among construction workers in this part of India (10).

A study was conducted at the University of Kansas Medical Center, USA to determine the prevalence of isolated and concurrent nickel, cobalt, and chromate sensitizations. A retrospective analysis was carried out on patch-test data of 1187 patients evaluated at one United States center from January 1, 1995 to December 31, 2004, 208 of whom had a positive reaction to at least one metal. The results serve to raise questions regarding occupationally related chromate allergy (11).

A study was undertaken by the Institute of Allergic and Immunologic Skin Diseases, Kolkata, India, to detect the epidemioallergologic pattern of footwear dermatitis in India. A total number of 155 cases with footwear dermatitis were evaluated from July 2005 to June 2006. The proportion of footwear dermatitis was 24.22% ($n = 155$) among a total of 640 patients patch tested during that period. Among the different categories of footwear allergens, the highest positivity was shown by leather and leather-related chemicals in 61.9% cases ($n = 96$) (12).

A recent Danish study showed a significant increase in the prevalence of chromate contact allergy after the mid-1990s, probably as a result of exposure to leather products. Data was analyzed from the period 1992–2009 at Odense University Hospital, Denmark, and it is observed that there is no significant change in the prevalence or exposure sources of chromate allergy during 1992–2009 were identified. Leather shoes (24.4%) were the most frequent exposure sources in chromate allergy, and were mainly registered in women, although the difference between men and women was not significant ($P = 0.07$). Cement and leather glove exposure occurred significantly more often in men than in women ($P = 0.002$). Foot dermatitis (40.3%) was the most frequent anatomical location, apart from hand eczema (60.6%) (13).

The Department of Dermatology and the Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, MN, USA, reviewed the results of patch testing with rubber allergens from January 1, 2000, through December 31, 2007. In total, 773 patients (64.2% female; mean age, 48.6 years) were patch-tested with a rubber series (27 allergens), and 739 (95.6%) were concomitantly patch-tested with a standard allergen series. Commonly affected sites of dermatitis were the hand (49.7%), foot (15.9%), leg (12.0%), and arm (10.9%). The most common occupations were health care worker (16.3%) and homemaker (6.5%); 11.3% were retired. The rate of allergic reaction to at least one rubber allergen was 245 of 773 (31.7%). The allergens that most commonly yielded positive reactions were 4,4-dithiodimorpholine 1% (28/286 [9.8%]), thiuram mix (56/739 [7.6%]), and diphenylguanidine 1% (57/759) [7.5%]. The analysis concluded that rubber is a frequent cause of ACD (14).

CONCLUSION

Overall, the evidence suggests a decreasing trend of allergic contact dermatitis with appropriate formulation changes following toxicologic and epidemiologic information. The EU Nickel Directive appears to have started to change the epidemiology of nickel allergy in Europe, but it should be revisited to better protect consumers and workers since nickel allergy and dermatitis remain very frequent (15,16). Contact allergy will likely always exist as humans may develop new allergy even to the substitutes used to replace the existing known allergens. When enlightened toxicologic and epidemiologic evidence based judgment (and appropriate regulation) are implemented, allergic contact dermatitis secondary to nickel, hair products, fragrances, chromate, and rubber additives, among other chemicals improves among the masses.

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57 Importance of the skin decontamination wash-in effect

Richard P. Moody and Howard I. Maibach

INTRODUCTION

As reviewed by Moody and Maibach (1), soap washing of skin conducted at 24 hours dermal exposure to environmental contaminants to wash off the nonabsorbed dose for in vitro dermal absorption tests may result in an increase in the amount of topically applied chemical detected in the receiver solution immediately following soap washing. The term "soap" is used generally here to refer to the wide range of cleansers used for skin washing/decontamination (e.g., soaps, detergents, surfactants, and solvents). Soap wash-associated enhanced percutaneous absorption has been termed the in vitro wash-in (W-I) effect in the Moody laboratory. Some, albeit scarce, in vivo data are also consistent with the W-I effect since Wester et al. (2) attributed significantly ($P < 0.05$) enhanced percutaneous absorption of hydrocortisone in urine from Rhesus monkeys ($n = 3$) with skin soap and water washing conducted at 24 hours versus monkeys tested without soap washing. Wester et al. (2) also reported that this "phenomenon" of soap wash-associated enhanced absorption was observed repeatedly with pesticides (unpublished data). Wester and Maibach (3) referred to an in vivo "washing-in" effect in reporting enhanced absorption of an unspecified herbicide in one Rhesus monkey. The W-I effect was explained in these early studies from the Maibach laboratory on the basis that skin washing conducted for the purpose of decontamination possibly enhanced the absorption due to surfactant and/or skin hydration effects (1–5). Other than several reports from the Moody laboratory that a W-I effect of various magnitudes was observed in vitro for several radiolabeled pesticides applied to the skin of different animal species including human (6–13), there is a paucity of W-I reports from other laboratories. Prior to considering the clinical importance and potential for adverse health effects of the W-I effect, the in vitro methods used need to be considered.

IN VITRO DERMAL ABSORPTION METHODS

The in vitro methods used in the Moody laboratory were detailed by Moody and Maibach (1). Briefly both the Teflon flow-through Bronaugh diffusion cells (0.64 cm^2 exposed skin surface area) and the flow-through aluminum automated in-vitro dermal absorption (AIDA) Moody cells (0.17 cm^2) were used to hold fresh viable dermatomed (0.5 mm thick) skin specimens (Table 57.1). The receiver solution pumped through the Bronaugh cells to simulate cutaneous blood flow was Hanks HEPES buffered (pH 7.4) balanced salt solution (HBBSS) with antibiotic gentamicin sulfate (50 mg/L) and 4% bovine serum albumin (BSA) added for tests with lipophiles. The Moody cells were perfused with Trizma buffered (pH 7.4) Ringer's saline with glucose (0.8 g/L) and the antibiotics penicillin G (60 mg/L) and streptomycin sulfate (100 mg/L) added, but did not use BSA since the "Bronaugh protocol" (14) was only adopted later by the Moody laboratory. The radiolabeled ^{14}C-chemical dose was applied either in acetone or in commercial product formulation (Table 57.1) to the skin surface exposed in the diffusion cells for 24 hours prior to soap washing, and receiver fractions were collected for up to 48 hours for analysis by liquid scintillation counting. For Bronaugh cell tests the outer stratum corneum skin surface exposed in each diffusion cell was washed at 24 hours using two successive Q-tip swabs with the first swab wet with 50% Radiacwash soap, a commercial product used for nuclear radioactivity decontamination obtained from Atomic Products (Shirley, New York, USA). A second swab was conducted with distilled water to rinse off the soap. In the Moody cell tests the skin surface was washed at 24 hours by a slow 5 mL/min rinse with 50% Radiacwash in a glass infusion syringe pump followed by a distilled water rinse. This methodology difference in skin soap washing is noteworthy since Moody et al. (10) considered this difference, coupled with a greater W-I effect for Bronaugh versus Moody cells in their study with DDT, suggested that the W-I effect was elicited by washing since the gentle mechanical friction/rubbing of the Q-tip swabs for Bronaugh cell tests may have sufficed to enhance the W-I effect. Besides further speculation as to the multiple possible causes of the W-I effect (Fig. 57.1), since the W-I effect was observed in receiver fractions collected immediately following the 24-hour skin soap wash, it is evident that this enhanced in vitro transdermal absorption was in fact elicited by the soap washing procedure (1).

Given the paucity of the in vivo W-I effect data, and that reports of the in vitro W-I effect have mainly come from the Moody laboratory albeit a few reports from other labs exist (1), the authors contend it is possible the in vitro W-I effect is at least partially an artifact of the in vitro methodology used (Fig. 57.2) as will be discussed under skin depot bioavailability. However, since the W-I effect was observed with different pesticides in different animal species of skin including hairless guinea pig, rat, pig, human, and human tissue cultured skins, and in both the Bronaugh and Moody cell tests, it is essential given the potential adverse toxicologic implications of the W-I effect, that further studies be conducted to verify/validate this effect in vivo. Prior to addressing such adverse health effects, the relevance here of skin depot bioavailability is discussed.

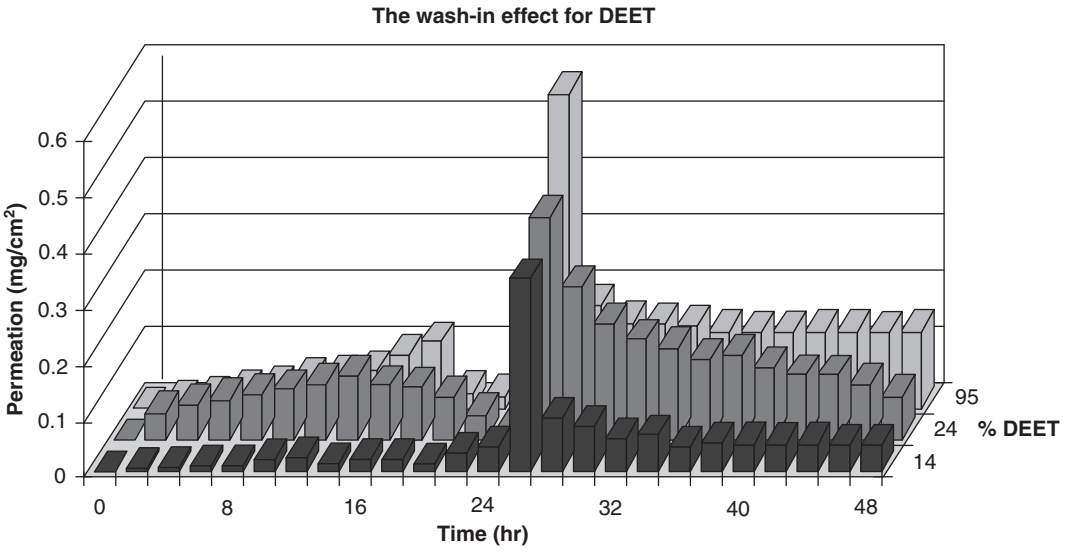

FIGURE 57.1 The wash-in effect for three diethyl-*m*-toluamide (DEET) insect repellent products in human skin tested in vitro. The z-axis shows the % DEET concentration in each commercial formulated product. Note the increased DEET levels in the receiver solution for all samples following the 24-hour soap wash of skin. *Source:* From Ref. 11.

TABLE 57.1
Previous Reports by Moody et al. of an In Vitro Wash-In Effect Listed Alphabetically by the Chemical Tested

Chemical	Vehicle	Cell	W-I effect	Study
Benzo[a]pyrene	Acetone	Bronaugh	Small and missing for some replicates	Moody et al. (12)
2,4-D acid	Acetone	Moody	Small	Moody et al. (9)
2,4-D amine	Formulated (two products)	Bronaugh	Strong for both products	Moody and Nadeau (13)
DDT	Acetone	Bronaugh	Strong	Moody et al. (10)
Diethyl-*m*-toluamide (DEET)	Acetone	Moody	Very small	Moody and Nadeau (7)
DEET	Formulated (three products)	Bronaugh	Strong for all three products	Moody et al. (11)
Diazinon	Acetone	Moody	Small	Moody and Nadeau (8)
Fenoxaprop-ethyl	Acetone	Moody	Not reported (only rat skin tested)	Moody and Ritter (6)

A subjective ranking of the relative strength of the wash-in (W-I) effect here is given in reference to the tests conducted with human skin except the study of Moody and Ritter (6) with fenoxaprop-ethyl which was only conducted with rat skin and reported no W-I effect. The relative strength of the W-I effect is given for each study cited and is, with exception of the FPE study, a subjective measure and is made in reference only to the human skin data. These studies report data for other test specimens including rat, hairless guinea pig, and tissue-cultured skin (Moody et al. (9) also used pig skin). Note overall that the W-I effect was stronger when formulated products were used, this suggesting a vehicle effect. The type of flow-through cell protocol (see text) used (Bronaugh Teflon or Moody aluminum AIDA cell) is also given. All tests used fresh tissues (unfrozen), except for some FPE tests (see text).
Source: From Ref. 1.

SKIN DEPOT BIOAVAILABILITY

The Organization for Economic Cooperation and Development (OECD) dermal absorption guidelines assert that unless it can be justified otherwise, the skin depot should be considered to be fully bioavailable (15). This international guideline is relevant since if the skin depot (also referred to as the skin reservoir (16,17)) could be liberated/mobilized by soap washing and yield a W-I effect, this potential for rapid release of the depot has both important toxicological and dermatotoxicological adverse health implications. Moody and Maibach (1) have previously considered that several mechanisms may underlie the W-I effect. These include effects of surfactants, skin hydration, pH (acid/base), friction/rubbing, and/or an artifact (Fig. 57.2). The latter explanation of an artifact relates to the artificially larger skin depot attained in vitro compared with that in vivo, especially for lipophiles (15), and is generally considered an artifact resulting from the use of a receiver solution which for very lipophilic chemicals may exceed the dissolution capacity (15,18–20). However, artificially large depots in vitro compared with smaller skin depots observed in vivo do not mean that less chemical should necessarily be predicted to be available systemically (i.e., percutaneously) from in vitro data. Higher in vitro depot levels/concentrations of contaminants still need to be accounted for when predicting in vivo absorption (15). The W-I effect is important since it can greatly accelerate transdermal delivery of depot/reservoir chemical resulting in higher receiver levels (i.e., blood levels) as exemplified by data for the mosquito repellent, diethyl-*m*-toluamide (Fig. 57.2).

Recently Nielsen (21) observed no W-I effect for four chemicals (benzoic acid, glyphosate, caffeine, and malathion) tested in vitro with human breast skin; however, skin washing was conducted at six hours which may not have been long enough to obtain a skin depot of sufficient magnitude to observe a

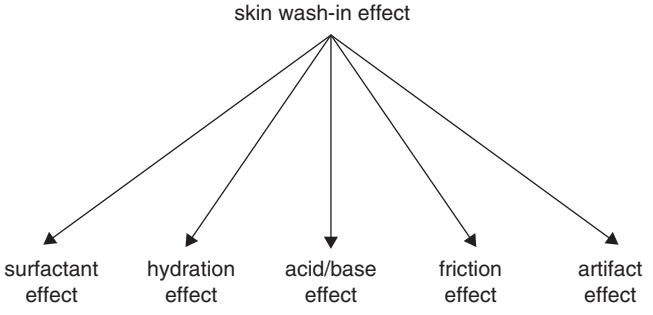

FIGURE 57.2 The mechanisms considered most likely to explain the wash-in effect. *Source*: From Ref. 1.

W-I effect. Nielsen (21) also used a pH neutral (pH 7.2) soap while the soap wash used by Moody et al. lab cited in Moody and Maibach (1) was acidic (pH 4.4, unpublished data) and a pH effect may partially explain the W-I effect (Fig. 57.2). Lademann et al. (22) reported on the basis of scanning electron microscopy, that the fluorescent dye fluorescein applied in a UV sunscreen to human forearm in vivo appeared to have been massaged deeper into the hair follicle by the mechanical stress of skin washing. Lademann et al. (23) also suggested on the basis of human in vivo forearm skin stripping data that skin washing may massage the sunscreen UV filter octyl methoxycinnamate deeper into the hair follicle and suggested washing could enhance transfollicular absorption since the inner human hair follicle has no stratum corneum protective barrier. The data of Lademann et al. (22,23) suggest that a partial explanation for in vitro W-I effect mechanisms (Fig. 57.2) may involve mechanical friction generated by washing at the skin washing Q-tip/skin surface interface. Mechanical friction enhancing skin depot hair follicle absorption is consistent with Table 57.1 data as other than benzo[a]pyrene with an at most small W-I effect, the data for Q-tip washed skin in Bronaugh cells showed strong W-I effects compared to at most small effects for the Moody cell, that was washed as described previously using a pump without Q-tip friction on skin.

HEALTH/CLINICAL IMPLICATIONS AND FUTURE RESEARCH

The W-I effect could in worst case scenarios increase dermal absorption to systemically acutely toxic levels and needs to be addressed in the clinical emergency exposure situation. An example is decontamination of first responders to chemical spills or chemical warfare agents released in terrorist incidents or the general public living in the immediate vicinity (24). Given the extensive use of chemicals in society that may contact skin such as those in cosmetics (25,26), and environmental contaminants such as those in soils from contaminated sites (27), it is important to understand the extent and potential health implications of the W-I effect. It is strongly recommended that further research be conducted to better understand the mechanisms underlying the W-I effect and its health implications. This should include investigating the effect of the time postexposure that soap washing is conducted since the maximum W-I effect may depend on time taken for the skin depot to become fully established.

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58 Water decontamination of chemical skin and eye splashes: Critical review

Alan H. Hall and Howard I. Maibach

INTRODUCTION

Burns of all types result in significant morbidity and mortality. Skin/eye chemical burns, perhaps better described as chemical skin/eye injuries because of the differences in pathophysiology from thermal or electrical burns, are a significant problem. More than 25,000 chemical products including oxidizing agents, reducing agents, and corrosives have been identified as having the potential to cause chemical injuries (1). The scope of the problem of chemical skin injuries is difficult to define, as there are no comprehensive reporting systems or structures for such injuries. Josset et al. (1974) reported that there were approximately 7000 serious occupational injuries annually from chemical burns in France, and that about half of these involved the eyes. These burns resulted in 120,000 lost workdays and 250 cases of permanent disability.

In a recent review, Palao et al. (2) note that while chemical burns only represent about 3% of all burns, such injuries have a significant morbidity with perhaps 55% requiring surgical treatment, commonly involve cosmetically disfiguring injuries to the face and chest, and are responsible for about 30% of fatalities in some case series (2). Disfigurement is particularly common in cases of deliberate chemical assaults (3,4).

According to an on-line fact sheet from the American Burn Association, approximately 3% of about 25,000 burned patients admitted to the 125 U.S. hospitals with specialized facilities for burn care ("burn centers") in recent years had chemical injuries (5). Some of the available sources which allow an approximation of the scope of the problem are described below.

The American Association of Poison Control Centers (AAPCC) has operated the National Poison Data System (NPDS; formerly the Toxic Exposure Surveillance System) since 1983. The NPDS collects information on poison exposures self-reported by telephone callers to poison centers serving the United States and its territories (6). Published data from the NPDS for the five years, 2006 through 2009, are summarized here.

Table 58.1 shows the total number of human poison exposures for each of the five years, the number and percent of all types of dermal exposures, and the number of fatal cases (6–11). During these same years, there were a total of 621,702 ocular exposures (or approximately 5.1% of total human poison exposures) recorded in the AAPCC NPDS database.

The AAPCC NPDS database has generally been thought to capture about 25% of all actual poison exposures in the United States. Because U.S. poison centers take telephone calls from the general public as well as healthcare providers, the NPDS database tends to over-represent asymptomatic and mildly symptomatic poison exposure cases. It tends to under-represent poison exposures in the workplace and also to under-represent fatal cases as there is no requirement or mechanism for coroners and medical examiners to report all fatal poisoning cases to the NPDS.

Because the NPDS does not differentiate in its overall summaries of the numbers of cases between exposures resulting in no symptoms and symptomatic exposures, especially by route of exposure, it is not possible to determine how many clinically symptomatic dermal or ocular exposures occurred during the reviewed five-year period. Also lacking in this database are detailed descriptions for most individual cases and important information such as the amount and concentration of substances involved in dermal exposures, delay to and type of decontamination measures utilized, and clinical outcomes such as lost work time, requirement for additional medical or surgical treatment, and sequelae.

Similar data are not currently available from the Canadian Association of Poison Control Centers. However, the most recent Annual Report from the Ontario Poison Center in Toronto notes that there were approximately 46,648 cases of human poison exposure reported to this Poison Center during 2009, of which approximately 6% (~2799) were dermal and 4% (~1866) were ocular exposures (12; see also 120).

The U.S. Department of Labor's Bureau of Labor Statistics collects data each year on nonfatal and fatal occupational injuries and illnesses (13). The most recent published data available at the time of this review are from the year 2009 (14). While these data are generally nonspecific and cannot easily be related to the epidemiology of chemical skin or eye injuries, they do provide some insight into the scope of the problem.

The total number of nonfatal workplace injuries and illnesses declined in 2009 to approximately 3.3 million cases (down from 3.7 million cases in 2008). This represented a decrease in the rate to 3.6 cases per 100 full-time equivalent workers in 2009 from 3.9 in 2008. The incident rates for injuries and illnesses also decreased significantly from 2008 to 2009, except for days-away-from-work (lost work time) cases whose rate was relatively unchanged from 2008.

Slightly more than one-half of the 3.3 million injury or illness cases in 2009 were of a more serious nature, involving lost work time, job transfer, or work restrictions. These latter occurred at a rate of 1.8 cases per 100 full-time equivalent workers. Cases of lost work time only were relatively unchanged from 2008 at 1.1 cases per 100 workers.

Approximately 3.1 million (94%) of the 3.3 million total cases in 2009 were injuries rather than illnesses. Chemical skin injuries would be included in the injury category. Chemical manufacturing would be an economic sector where chemical skin injuries would

TABLE 58.1
Total Number of Human Poison Exposures

Year	Total Poison Exposures (No.)	Total Dermal Poison Exposures (No.)	% Dermal Poison Exposures (%)	Fatal Dermal Poison Exposures (No.)
2005	2,424,180	194,954	7.70	14
2006	2,403,536	188,848	7.50	21
2007	2,482,041	191,298	7.30	14
2008	2,491,049	188,930	7.22	22
2009	2,479,355	179,832	6.90	12
Total	**12,280,161**	**943,862**	**7.60**	**83**

be expected to occur. Of nonfatal occupational injuries and illnesses reported in 2009, in the chemical manufacturing sector, the rate was 2.3 per 100 workers with a rate for lost work time injuries and illnesses of 0.6 per 100 workers. In actual numbers of cases, there were a total of 20,000 cases in the chemical manufacturing sector, of which 5400 were lost work time cases.

The rate of nonfatal occupational injuries in the chemical manufacturing sector was 2.1 cases per 100 workers, representing 18,100 total cases. Chemical skin injuries would be expected to be included in the incidence rate for skin diseases and disorders. For all industries in 2009 (including state and local governments), this rate was 3.4 per 10,000 full-time workers.

Fatal occupational injuries in 2009 comprised 4340 cases. Of these, fatalities due to exposure to harmful substances or environments comprised 390 cases (9.0% of the total) in all industries and 357 cases (8.32% of the total) in private industry. Of the total fatalities, the chemical manufacturing sector had 18 cases, but none of these were due to exposure to harmful substances or environments. On a list of fatal occupational injuries by source, there were 129 cases listed as being due to chemicals and chemical products (which in addition to corrosive substances, includes toxic gases and vapors, medications, alcohol, drugs, etc.). Of the total occupational fatalities in 2009, 119 cases (30.5%) of the total 390 cases due to exposure to harmful substances or environments were due to exposure to caustic, noxious, or allergenic substances.

Occupations and operations with 20 or more fatal occupational injuries in 2009 due to exposure to harmful substance or environments are shown in Table 58.2 (14).

The American Burn Association collects all types of burn cases reported by member hospitals in its National Burn Repository (15). The National Burn Repository Report Data from 1999 to 2008 cover a total of more than 220,000 patients treated by contributing hospitals. Over the years, chemical burn injuries have accounted for approximately 3% of all burn injuries in this dataset. During the same time period, data analysis of 1938 cases contributed by three Canadian and one International burn centers showed 64 chemical burn injuries (3.4% of total cases).

The 2008 report covers a total of 127,016 patients with burn injuries, of which 2494 had a chemical etiology. While workplace chemical skin injuries are not specifically tracked in the Repository, of the total number of burns (all etiologies) 11,933 (11.1%) occurred in an industrial setting and 14,119 (15.2%) of cases were the result of a work-related accident.

There was a greater percentage of chemical burn injuries listed by age groups in patients aged 16–69.9 years (the ages at which it might be expected that many persons would be employed) (Table 58.3).

TABLE 58.2
Total Number of Fatal Occupational Injuries from Exposure to Harmful Substances or Environments, 2009 (n = 390)

Occupation/Operation	No. of Cases	% of Total Cases
Building and grounds cleaning and maintenance operations	45	11.5
Farming, fishing, and forestry operations	20	5.1
Construction and extraction operations	125	32.1
Installation, maintenance, and repair operations	57	14.6
Production operations	29	7.4
Transportation and material moving operations	35	9.0

TABLE 58.3
Chemical Burns by Age Group

Age Group (years)	No. of Chemical Burn Injuries	% of Total Burn Injuries
Birth–0.9	32	1.0
1–1.9	82	1.0
2–4.9	54	0.8
5–15.9	87	0.8
16–19.9	132	2.4
20–29.9	541	3.7
30–39.9	657	4.9
40–49.9	660	4.6
50–59.9	360	3.7
60–69.9	169	3.1
70–79.9	43	1.1
80 and above	28	0.9

In the pediatric population, chemical skin injuries contribute only a minor portion of burns, with thermal injuries being most common in this age group (16).

As per the data collected by the U.S. Consumer Product Safety Commission in its National Electronic Injury Surveillance System (NEIHS) database, between the years of 1990 and 2006, there were an estimated number of 2,054,563 patients aged ≤20 years treated for burn injuries in U.S. Emergency Departments (17). Actual cases of chemical burns were 4792 with national estimates being 180,652 cases, or 8.8%. Of consumer products, chemicals and cleaners accounted for 3944 actual cases and 148,126 estimated cases, or 8.6% (17). In this population (</= 20 years of age), chemical burns were 2.5 times more likely to occur at schools, sporting recreational facilities, or in other public settings rather than in the home (17).

In a questionnaire survey distributed as part of an assessment of the ability to manage chemically-contaminated patients in the United Kingdom, the questionnaire was sent to trainers and department heads in all 224 acute hospital and ambulance trusts (64). This comprised 192 hospitals and 32 ambulance trusts; 49/192 hospitals (26%) and 6/32 ambulance trusts (19%) responded. Among the 6 responding ambulance trusts, the mean number of chemical incidents dealt with in the previous 12 months was 74

(range: 0–371). For hospitals, the mean number of chemical incidents over the same time period was 1 (range: 0–4) (64). How many of these chemical incidents involved skin exposure or decontamination was not reported by these authors.

Following the removal of contaminated clothing which has been said to decrease chemical skin decontamination by up to 80% (18), standard references recommend water or normal saline (0.9% sodium chloride solution) for immediate decontamination of skin/eye chemical splashes, with the addition of soap or a mild detergent if the chemical substance is lipid soluble (18–22). Soap or detergent should not be used in the eyes. Older literature suggests that immediate flushing of the eyes for about 30 minutes from the nearest shower or faucet should be done following sodium or ammonium hydroxide ocular exposure (23).

In the United States, the Occupational Safety and Health Administration (OSHA) regulations mandate emergency eyewash stations and quick-drench showers in all facilities where potential dangerous chemical agents are present (1). Most of these facilities utilize water for skin eye decontamination. Current recommendations for water decontamination of chemical splashes can be found in the ANSI/ISEA Z358.1–2009 American National Standard for Emergency Eyewash and Shower Equipment and the European Norms (European Committee for Standardization NF EN 15154–1; 2006).

A real concern has been raised about water decontamination of chemical skin splashes, the "wash-in effect" (24). Data reviewed in this publication suggest that, at least with some contaminants and in some circumstances, water washing may actually increase dermal systemic absorption and enhance systemic toxicity (24).

MATERIALS AND METHODS

In-depth electronic and paper literature searches were performed to retrieve pertinent articles and reports involving water decontamination of skin/eye chemical splashes and chemical burns/chemical injuries. Searches were done in the U.S. National Library of Medicine PUBMED and TOXNET databases using combinations of search terms such as "chemical burns," "skin burns," "dermal burns," "eye burns," "ocular burns," "occupational burns," "workplace burns," "chemical decontamination," "skin decontamination," "eye decontamination," and "ocular decontamination." For older literature, hardcopy versions of Index Medicus were reviewed back to 1929. The references/bibliography section of each retrieved paper was also reviewed for any pertinent references.

Organization web pages, such as that of the American Burn Association, were reviewed for data on the occurrence and etiology of chemical burns/chemical injuries, and for references/links to other sources of chemical burn/chemical injury data such as State Health or Health and Environment departments. These web pages were also reviewed. Chemical burn/injury occurrence and outcome data from the U.S. Department of Labor-Bureau of Labor Statistics website were also reviewed. Internet Google searches were also conducted using all combinations of the above search terms.

RESULTS

One estimate stated that approximately 5511 deaths were associated with fire in burns in the United States in 1991 (25). Of these, 125 deaths were said to be due to hot liquids, substances, and objects (including caustics and corrosives) and contact with these accounted for nearly 500,000 emergency department visits (25–27). In a 1977 University of Michigan Quality of Employment Survey of 36 illnesses and injuries and 17 job hazards in 1515 workers, chemical burns were in the top three illness and injury categories among employed men (28). While chemical burns account for only about 2–5% (29–39) of all burn injuries, they are responsible for over 30% of burn deaths (32).

Chemical assault injuries are an important subtype of chemical skin injuries, perhaps particularly in developing countries (40,3). Nitric and sulfuric acids are commonly involved, and children and young women may be at particular risk (40,3).

Occupational Burn Information Collected by Governmental Agencies or Assembled From Government Sources

West Anglia and Oxford Region, UK

In four U.K. counties in the West of Anglia and Oxford region, Wilkinson (41) reviewed the epidemiology of burn patients treated in accident and emergency departments or admitted to the hospital for burn care during 1994–1995. About half of the burn patients admitted to the hospital were treated in burn units, one-fourth were treated on plastic surgery wards, and the remainder were admitted to specialty units including trauma, orthopedics, pediatrics, and ophthalmology. The largest numbers of admitted patients were in the working age group, which was also the largest group in the general population. Burn patients accounted for approximately 1% of cases seen in accident and emergency departments and about 10% of these patients were admitted to the hospital, with a mean length of stay of 7.5 days (41).

South Wales, UK

Munnoch et al. (43) studied the work-related burns in South Wales during a two-year period between 1995 and 1996. There were 324 cases of work-related burns and records were available for 319 of these. Twenty percent of all burns referred to the burn center in Swansea occurred in the workplace. Chemical burns were the most frequent cause (23%) with caustic soda (sodium hydroxide) exposure in 21 cases, cement in 15 cases, and various acids and alkalis accounting for the remaining 37 cases. Of these 319 patients, 175 were admitted to the Burn Centre and 79 required surgery. The mean length of hospital admission was 8.5 days (range 1–110 days), representing overall 1485 hospital days. Men aged 16–40 years comprised 70% of the patients with work-related burns. Fifty-five percent of patients with work-related burns were admitted to hospital and approximately 25% required surgery (43).

Switzerland

De Roche et al. (44) studied the epidemiology and cost of work-related burn injuries in Switzerland. They noted that about 4.6% of all accidents in Switzerland were burns and that 3.0% of all accidents were work-related burn injuries. Estimates based on population suggest that there were 36,000 burns annually in Switzerland with 5% requiring hospital admission and one-third of these being treated in a burn center. There is a compulsory insurance program for workers in Switzerland that covers accidents both on and off the job. In 1984, 6814 burn accidents were covered by this insurance program, 58% work-related and 42% non-work-related. The

total cost for burns was 17.7 million Swiss Francs, with 19% for medical care, 34% for salaries while off work, and 46% for annuities (44). The proportion of chemical burns was not reported.

Taiwan

Chien et al. (45) studied the epidemiology of hospitalized patients with burns in Taiwan during a 2-year period from 1997 to 1999: a total of 4741 patients were hospitalized for burn treatment. Work-related burns occurred in 1459 patients (30.8%). Among adult patients, chemical burns due to exposure to corrosive agents such as strong acids or alkalis accounted for 9% of the injuries. Burns due to explosions and chemical contact occurred more frequently in the workplace (32.9%), were more serious (average 25% total body surface area [TBSA]), and resulted in longer average hospital admission times (23 days) (45).

U.S. NIOSH/CPSC

Over 3 months in 1981, the U.S. National Institute for Occupational Safety and Health (NIOSH) and the Consumer Product Safety Product Safety Commission (CPSC) conducted a surveillance program of occupational injuries treated at a sample of 66 U.S. hospital emergency departments (46). There were a total of 2747 burn injuries (cause not specified) that represented 4.5% of the total 61,585 occupational injuries treated in these facilities. The most common sites of burns were face, arm, and trunk. Based on the surveillance system, it was estimated that there were 3.3 million occupational injuries treated in emergency departments in the United States in 1981 (46). If the percentage of burns were constant, this would represent 148,500 occupational burn injuries yearly. Chemical burns were not listed separately from all occupational burns.

In a retrospective analysis of National Hospital Ambulatory Medical Survey databases from 1996 to 2005 available from the U.S. Centers for Disease Control and Prevention, Taira et al. noted amongst burn patients admitted to Emergency Departments in the United States, the etiology of "caustic/corrosive substances" was the fifth most common and accounted for approximately 3% of total burn injuries (47).

New England, USA: Rossignol et al. (48) collected data on burned patients aged 20 years or older admitted to any of 240 of New England's 256 acute-care hospital for treatment of a burn injury. Chemical burns were among the type of injuries identified in the study. Overall, 1614 new burn injuries were identified during the one-year study period between 1978 and 1979. Of these, 485 burns (30%) were work related. Overall, 40% of the 1133 burns in men were work related, whereas only 7% of the 481 burns in women were work related. There were 91 chemical burns in men, of which 67 (74%) were work related.

State of North Carolina, USA

Hunt et al. (49) in a survey of occupation-related burn injuries during 1994 using data from the U.S. National Census of Fatal Occupational Injuries (fatal cases) and the North Carolina Department of Labor (nonfatal cases) found that there were 34 burn deaths (15.3%) and 1720 nonfatal burns. Burn injury was the fourth most common cause of workplace deaths, but what proportion of these were chemical burns was not specified. Of the nonfatal burns, 709 (41.2%) were caused by chemical exposure. Involved chemicals were alkalis (20%), cleaners and solvents (16.9%), propane (12.2%), halogens (7.0%) inorganic and other acids (3.6%), hydrocarbons (2.0%), and other chemicals (38.3%). Chemicals and chemical products were the most common agents causing workplace burn injuries (49).

State of Washington, USA

In a study of occupational burns in Washington State during a five-year period from 1989 to 1993, there were 27,323 workers' compensation claims for work-related burns; 7323 (26.8%) of these were chemical burns (50). Of exposures, 2173 (8%) were related to an unspecified chemical, 906 (3.3%) were to soaps and detergents, 604 (2.2%) to solvents/degreasers, 462 (1.7%) to calcium hydroxide, 451 (1.6%) to chlorine compounds, and 371 (1.4%) to sodium hydroxide. Industries at the highest risk for chemical burns were hazardous waste landfill cleanup, portable cleansing and washing; pulp and paper manufacturing; and chemical blending, mixing, and manufacturing (50).

Baggs et al. (51) investigated work-related burns in Washington State during 1994–1998. There were a total of 20,213 work-related burn claims accepted by the workers' compensation system during this period, but only 1.5% of burned workers were admitted to the hospital. However, this 1.5% of burned workers represented 55% of the cost incurred. The costs for all work-related burns were in excess of US$5 million annually. Patients hospitalized for work-related burns lost an average of 132 workdays, while burned workers not requiring hospitalization lost an average of 3 workdays. These authors noted that the workers' compensation data underestimated the frequency and cost of work-related burns (51). Burns were evaluated in two categories: thermal and chemical. Among previously identified high-risk industries were hazardous waste clean-up and the chemical industry. In this study, chemical mixing and manufacturing, concrete work, and construction ranked high as industries having hospitalized work-related burn cases; janitorial services was also an industry having chemical burn cases reported which had not been identified in previous studies (51).

State of Utah, USA

The Utah Department of Health Bureau of Epidemiology collected data on work-related burns during 1997 (52). In 1997, there were 699 hospital admissions for burn treatment, of which 133 were work related. Male workers accounted for 82% of these cases. Workers aged 24–44 had 60% of all work-related burns (52).

State of Colorado, USA

The Colorado Department of Health and Environment noted that over a period from 1980 to 1989, an average of 24 state residents died each year from burn injuries (53). Approximately 330 Colorado citizens were hospitalized yearly for burn injuries, and approximately half of these were due to scalds, hot objects, or exposure to caustic substances (53).

State of Massachusetts, USA

Rossignol et al. (54) studied the epidemiology of work-related burn injuries in Massachusetts necessitating hospital admission during a one-year period in 1978–1979. Of the total 825 total burn

admissions, 240 (29%) were work related. Of the total work-related burns, 95% were in men. There were 29 chemical burns that accounted for a total of 248 hospital admission days.

State of Ohio, USA

Chatterjee et al. (55) studied 199 burn injuries in northeastern Ohio evaluated in an emergency department during 1977, representing 2.4% of all patients evaluated for any type of trauma. The cause of the burn was known in 187 cases (94%). Of these, 124 (66%) were due to hot substances, corrosive liquids, or steam (not further delineated). Of these patients, 55 had a work-related burn and 52 claimed eligibility for workers' compensation.

Burn Center/Unit Data without Information on Decontamination and Clinical Outcome

France

A review of survival rates in hospitalized French burn centers during 1985 was performed by Wassermann and Schlotterer (56). A total of 2398 patients were admitted for treatment to 17 French burn centers and there were 238 deaths, for an overall mortality rate of 11.8%. Young adults constituted the majority of admissions (>50%) and the authors noted that the withdrawal of these persons from economic activity for weeks or months had a significant impact (56).

Hobart, Tasmania, Australia

Ricketts and Kimble (57) reviewed 31 patients with chemical burns admitted to a burn center in Hobart, Tasmania between 1989 and 1999. Of these, 38% occurred in an industrial setting and the most common chemicals involved were cement (25%), sulfuric acid (16%), and hydrofluoric acid (16%). The mean TBSA affected was 3.5% and the mean hospital admission time was nine days (range 1–30 days).

Brisbane, Queensland, Australia

Pegg et al. (58) reported a series of occupationally-related burn cases admitted to a hospital in Brisbane, Queensland over a 7-year period between 1976 and 1983. There were 182 occupationally-related burn patients, of whom 95% were men. There was one fatality. Half of these burns occurred in men less than 30 years old. Ocular burns were present in 5.5% of these patients and were caused by chemical exposure, gas explosions, and electrical flashes. Eighteen patients in this series had chemical burns that were less than 30% (mean 2.25%) of the TBSA. Sulfuric acid and caustic soda (sodium hydroxide) were the most common causes of chemical burns. Perchloroethylene, ammonia, cresylic acid, hydrofluoric acid, and Shellite (white gas; Coleman fuel: a refined petroleum product) also caused chemical burns. The eyes and hands were the main burn sites (58).

Adelaide, South Australia, Australia

In a retrospective study of 1584 acute adult burn injury patients between 1996 and 2004, chemical burns accounted for 5.8%, with predominantly liquid acid and alkali injuries (59). The involved TBSA ranged from <1 to 65%, but with most patients having <10% of the TBSA affected (59).

Perth, Western Australia, Australia

In an epidemiologic analysis of 5398 children aged less than 5 years admitted to hospital for burns between 1983 and 2008, the etiology was chemical/corrosive injury in 129 (2.4%) (60). Three children had full-thickness chemical injuries and 24 had partial-thickness injuries. The median length of hospital stay was 1 day (range: 1–4 days) (60).

Curacao, Netherlands Antilles

In a retrospective study of 336 patients admitted to a burn center between 1992 and 2002, chemical burns were the etiology in 16 patients (5%) and men had the majority of such injuries (61).

Calabar, Southeastern Nigeria

In a prospective study of burn patients admitted to a University teaching hospital in Nigeria between 2005 and 2008, chemical injuries were the second most common etiology, involving 7/59 (11.9%) of patients (62). Chemicals involved were acids in six cases and alkali in one case. Deliberate chemical assault involved robbery (two cases), and a love affair or conflict (two cases). Seven of the 59 patients used cold water for first aid, but the authors did not report whether these were the chemical injury cases as opposed to thermal injury cases. Complication reported were wound infection (5/7 chemical injuries) and contractures (4/7 chemical injuries) (62).

Kwa-Zulu Natal, South Africa

In a prospective study of 450 burn patients admitted to a regional hospital in South Africa between 2006 and 2008, the etiology was chemical exposure in six cases (1%), all of which were adult victims of deliberate chemical assault (63).

Dammam, Saudi Arabia

In a prospective study of hospital burn admissions, chemical injuries accounted for six (8%) of 240 total cases between 1997 and 2003 (65).

Copenhagen, Denmark

In a study of occupational burn injuries treated in the municipality of Copenhagen, Denmark during a one-year period from 1983 to 1983, 371 patients had work-related burn injuries (66). Of the 361 patients treated in casualty wards, 70 were subsequently treated in the burns unit, six as inpatients and the rest as outpatients. There were 24 patients with corrosive chemical injuries, 18 of which (75%) occurred in the chemical industry. The involved chemicals were alkalis 13, acids 7, and other chemicals 4 (66).

Kuopio, Finland

Among the first 1000 inpatients treated at a University Hospital Burn Center between 1994 and 2006, there were 104 (10.4%) work-related burns (67). Alkali injuries accounted for 2% of these patients and acid injuries for 1% (67).

Cairo, Egypt

Nasser et al. (68) reported a retrospective review of 549 children aged 2 months to 15 years treated in a pediatric burn unit during

a 12-year period from 1996 to 2007 (68). Chemical burns were the etiology in eight patients (1.4%). These authors noted that 280 of the children admitted to the burn unit were victims of child labor industrial accidents and that five of them had chemical burns (68).

Tel-Hashomer, Israel

In a retrospective study of 2705 children aged 0–14 years and hospitalized for a burn injury in all five burn units in Israel between 1998 and 2004, chemical burns were the etiology in 3% (69). A second analysis of 5000 consecutive burn patients admitted for at least one day to one of the five major hospitals with burn units in Israel during a seven-year period from 1997 to 2003 found that chemical injuries were the etiology in 6% of patients (70).

Toronto, Ontario, Canada

Ng et al. (38) performed a six-year retrospective study of 193 work-related burns in patients treated in a burn center in Toronto, Ontario between 1984 and 1990. Of these, 94.3% were men and 64.2% were under the age of 35 years. Chemical injuries accounted for 5.1% (10 cases) of the total. Chemicals involved were alkalis (sodium hydroxide, "detoxification agent", pulp decomposing agent, and unknown; six cases) and acids (hydrofluoric acid, sulfuric acid, and phenol; four cases). The mean TBSA chemical burn was 6.0% (range 1–98%). The total cost of hospital treatment for these 193 work-related burns was Can$ 296 million and the estimated time lost from work was 439 workdays (38).

A follow-up study from this same burn center described 100 cases of occupationally-related burns admitted between 1988 and 2000 (71). Of these, chemical injuries were 7% of the total. No changes were noted in the incidence or severity of work-related burn injuries from the earlier study by Ng et al. (38) and the authors remarked that the preventive measures undertaken over a 10-year period were not effective (71).

New Delhi, India

In a continued analysis of burn admissions over the years 1993–2000 (11,196 cases) compared with 2001–2007 (5,666 cases), there was a significant change in the epidemiology profile with chemical burns increasing from 45 (0.41%) in the earlier period to 125 (2.25%) in the latter period (72).

Tehran, Iran

Lari et al. (31) reviewed 3341 burn patients admitted to a burn center in Tehran, Iran during a three-year period between 1995 and 1998. Of the 110,554 patients evaluated, 3341 (3%) were admitted to the burn center. Of the admitted patients, 124 (3.7%) incurred the burn in the workplace. A total of 67 burns (2%) were due to chemical exposure.

In a cross-sectional epidemiologic study of 4813 patients treated as outpatients in a referral burn center in Tehran, Iran during a one-year period in 2004, most burns were not intentional, although three were the result of deliberate chemical assaults (73). There were 66 (1.4%) chemical burn injuries with the majority (53/66; 80%) occurring in patients aged 19–60 years when many would have been expected to be employed. Upper extremities were the most common site of chemical injuries (73).

Hamadan, Iran

In a retrospective study of pediatric burns (patients aged 0–14 years) hospitalized in a regional burn center in Iran, children comprised 29.3% of total admissions and the mean age was 4.58 years with 69% of patients less than 4 years old (74). The total fatality rate was 3.5%. Chemical skin injuries were not specifically listed under etiologies in this study, but the "other" category comprised 0.6% of total pediatric burn injuries (74).

Chennai, India

In a study of 1368 patients evaluated for burns in a Medical Hospital in Chennai, India during a one-year period from 1987 to 1988, there were 135 work-related burns (75). Of these, 18 were due to chemical exposures.

Digboi, Assam, India

Sarma and Sarma (39) performed a retrospective study of 348 burn patients admitted to a peripheral industrial hospital in Digboi, Assam, India during a 10-year period between 1980 and 1990. Overall, work-related burns comprised 12% of the study group. There were 20 chemical burns (5.7%) from exposure to acids and alkali. Of 42 work-related burns, five were due to acid exposures (39).

Chongquing, China

A retrospective study of 280 burn patients older than 60 years admitted to a burn unit during 1999–2006 represented 4.15% of all burn patients admitted during this period (76). Chemical burn injuries accounted for 3.6% of total burn cases in these more elderly patients (76).

Istanbul, Turkey

In a retrospective review of 358 children aged 0–14 years admitted to a Turkish burn center over a 7-year period of 2001–2008, chemical burn injuries comprised 1.52% (77).

San Diego, California, USA

In a study of 232 cases of all types of occupational burns treated in a burn center in San Diego from 1977 to 1982, chemical burns accounted for 4% of all patients (37). Of the admitted chemical burn patients, 50% were permanently disabled, the median hospital stay was 12 days, and the median time from hospital discharge to return to work was 13 days in those who were not permanently disabled. While chemical burns were not common, they were often severe (37).

Atlanta, Georgia, USA

In a study of 844 burn center admissions in Atlanta over approximately 3.5 years from 1987 to 1990, there were 33 chemical burns (3.9%) with a mean TBSA of 9.0% and an overall survival of 90.9% (34). There were three deaths from chemical burns.

Shanghai, China

A retrospective study was conducted of the characteristics of burn patients discharged from a burn unit in Shanghai between 2002

and 2003 (78). Of the 527 included patients, the majority of burns (58%) were work related. Of all etiologies, chemical exposure comprised 7%. Among adults, chemical exposures accounted for 9.5% of the injuries and most burn injuries occurred in the workplace (78).

Burn Center/Unit Data with Information on Decontamination and/or Clinical Outcome

Toronto, Ontario, Canada

In a review of chemical burn patients admitted to a regional burn center in Toronto, Ontario over an 8-year period, 24 chemical burn patients comprised 2.6% of all admissions (30). Work-related accidents accounted for 75% of these burns, with the involved chemicals being hydrofluoric acid, sulfuric acid, black liquor (a heated mixture of sodium carbonate, sodium hydroxide, sodium sulfide, sodium thiosulfate, and sodium sulfate), various lyes, potassium permanganate, and phenol. Of these 24 patients, 14 required extensive excision and skin grafts. Complications were frequent (58% of patients), including eye contact with the chemical, wound infections, tendon exposures, toe amputation, and systemic toxicity from chemical absorption. One patient with a chemical scald burn involving 98% of the TBSA died. In 14 of 24 patients (58%), removal of contaminated clothing followed by immediate water shower decontamination was done; five other exposed patients did not have these interventions. Five of the eight eye splash cases had immediate decontamination at the site (presumably with water). While the three eye splash patients who did not have immediate decontamination developed prolonged conjunctivitis, three of the five who were decontaminated immediately developed corneal erosions and one who had eye exposure to back liquor had a very deep corneal erosion, which resulted in blindness (30). Despite immediate decontamination of the skin or eyes with water, some of these patients developed burns and significant complications.

Chandrigarh, India

In a study of 27 cases of acid and alkali burns evaluated over a five-year period, Sawheny and Kaushish (79) noted that chemical injuries differed from thermal injuries. Of 562 patients admitted to a burn center over a five-year period, 16 were acid exposures (sulfuric or nitric acids) and 11 were caustic soda (sodium hydroxide) exposures. The 11 patients exposed to caustic soda were involved in the collision of a tank truck with a passenger bus. The majority of chemical burns (20/27; 74%) involved less than 15% of the TBSA and 81.5% were full-thickness burns; mainly on the face, upper trunk, arms, and hands. Eye involvement was present in 74% of patients and both eyes were involved in 15%. Severe conjunctivitis was present in all patients with eye exposure, with keratitis and corneal ulcerations progressing to opacities occurring in 63% of patients. Corneal perforation progressing to panophthalmitis and vision loss occurred in two cases. Severe eyelid ectropion developed in 12 patients (44%). By the end of the third week, wound infections developed in two-thirds of patients and all wounds were infected by 4 weeks after surgery. Invasive sepsis occurred in one patient. In these patients a "...thorough and continuous irrigation of the area of damaged tissues with copious volumes of water... was done as early as possible"..., although this may have been after a delay of hours following exposure, but was noted to be of limited effectiveness when the patients did not arrive until a delay of days (79).

Tabriz, Iran

In a prospective study of 121 chemical burn cases treated over a 5-year period from 2001 to 2006, the male:female ratio was 10:1 and the average TBSA was 7.98% (80). The most frequent chemical injury patients were young men with work-related injuries. Of the total burn cases, 78.5% occurred in the workplace, 111 (91.7%) were accidental, and 10 (8.3%) were deliberate chemical assaults. The mean hospital stay was 10 days (range: 1–62 days). The mortality rate was 1.7%. Etiologic agents were strong acids (sulfuric acid, nitric acid, and hydrofluoric acid) (22.3%), alkalis (mainly lime and sodium hydroxide) (4.1%), tar (60.3%), Tyner (2.5%), and cement (0.8%). The most frequently injured areas were the upper limbs (51.6%) and lower limbs (31.6%). Complications included bilateral or unilateral blindness (3 cases), neck contraction (5 cases), loss or deformity of the external ear (2 cases), bilateral or unilateral finger contractions (6 cases), and facial deformity (7 cases). The prevalence of chemical injuries amongst all burn patients during this time period was 2.4%. Two deaths occurred in patients with 30 and 45% TBSA chemical injuries. The injuries, complications, and fatalities occurred despite initial large-volume water decontamination (80).

Sri Lanka

In a cross-sectional retrospective review of a burns registry in Sri Lanka over an 18-month period from January 1, 2008 to June 30, 2009 for all acid assault victims, of a total of 1134 burn patients, chemical burns represented 7% (n = 80), and 70 (87.5%) of these patients had acid injuries (4). Acid assaults involved 46 patients (4% of all burn patients; 57.5% of all chemical burns). In this study, the male:female ratio was 2.8:1. Of these acid assaults, formic acid was involved in 19 cases (41.3%), nitric acid in 1 case (2.1%), and unknown acid in 26 cases (56.5%). Prehospital decontamination with water was done in 26 cases (56.5%) and not done in 20 cases (43.5%). The mean TBSA was 14.6% (range 1–50%) with the most commonly involved areas being the face (93%), chest (65%), and upper limbs (64%). There were two fatalities. Long-term sequelae included hypertrophic scars with constriction bands, ectropion, and microstomia, and five (10.8%) patients required surgery for scar revision (4).

Irrua, Nigeria

In a retrospective study of 72 patients admitted to a specialist teaching hospital during a 12-month period in 2006, there were four (5%) chemical burns, all of which were acid injuries (81). Of these, one case involved a battery charger accident and the other three were deliberate chemical assaults. One of these patients died. There was "little proof" of first aid water washing and the authors noted that "...the environment lacked running water" (81).

Gaziantep, Turkey

In a retrospective analysis of 411 cases admitted to a burn center during the period of May 1, 2007 to October 10, 2008, chemical burns resulted in a mean hospital stay of 33 days (82). Chemical

burns comprised 4.3% of total burns, the fourth most common etiology. With an involved TBSA of 10–20%, those patients with chemical injuries had a mean hospital stay of 10 days, while this increased to a mean hospital stay of 33 days with 35–50% TBSA chemical injuries (82).

Bradford, UK

In a prospective study of all patients (n = 460) seeking care for burns at a single U.K. Emergency Department during a one-year period from 2003 to 2004, 88.5% of cases were accidental in nature and 13% were work related (83). Chemical injuries accounted for 6% of total burns. First aid by irrigation with cold water was reported in 51% of total cases, but chemical exposures irrigated with water were not specifically described by these authors (83).

Boston, Massachusetts, USA

In a study of 957 inpatients treated at a burn center in Boston during a four-year period from 1976 to 1980, 4% had chemical burns (35). The chemicals involved were acids (sulfuric, hydrochloric, hydrofluoric, carbolic [phenol], chlorosulfonic, and trichloroacetic; 15 cases), alkalis (lye and cement; 9 cases); and other/unknown substances (10 cases). The mean TBSA burn was 8.7%, mortality was 6% (2 patients), and the mean length of hospital admission was 15 days. The injury occurred in the workplace in 18 cases (51%), in the home in 10 cases (29%), and was due to a deliberate chemical assault in seven cases (20%). Sixteen patients had immediate water decontamination (within 10 minutes of exposure and lasting at least 15 minutes) and 19 had delayed water decontamination. The delayed decontamination group had a five-fold greater incidence of full-thickness burns and a significantly longer duration of hospital admission, despite the fact that the mean TBSA burn in the delayed decontamination group was a half that of the immediate decontamination group (35). Although immediate water decontamination appeared to decrease burn severity, it was unable to completely prevent development of burns and 16 patients in this category still required hospital admission for a mean of 7.7 days and 12.5% of them had full-thickness burns.

Iowa City, Iowa, USA

In a review of patients with chemical burns admitted to a burn center, of a total of 2762 patients, 94 (3.3%) had chemical burns (34). Of chemical burn patients, 31 (34%) were due to anhydrous ammonia. Chemicals involved included acids (14 cases), bases (68 cases), inorganic agents (2 cases), organic agents (6 cases), and unknown (5 cases). The majority had work-related burn injuries. Patients either had immediate water decontamination at the incident site or underwent water flushing in the burn center until the skin pH returned to normal. Further water irrigation was done if discomfort recurred and daily hydrotherapy was performed. There was one fatality in this group, and 36/94 (38%) required skin grafting with five patients having multiple procedures. Complications including would infections, pneumonia, cardiac failure, cardiac arrhythmias, and myocardial infarction occurred in 24/94 (25.5%), and sequelae were noted in 27/94 (28.7%) (34). Early and prolonged water decontamination did not prevent serious burns from developing.

Experimental Animal Studies

Older experimental animal literature supports immediate and even prolonged (up to 8 hours) continuous irrigation with water for acid or alkali chemical burns, noting that the sooner it is begun, the more likely it is to be effective (84–86). Delays to initiation of water decontamination of as little as 5–30 seconds sometimes make significant differences in its ability to prevent or decrease the severity of burns (85,86). Of note, statistical comparisons of various decontamination modalities are generally lacking in these older studies.

One of the earliest series of experimental animal studies was by Davidson (84) who investigated acid (50 and 70–71% nitric acid; 10, 25, 50, and 96% sulfuric acid; 37% hydrochloric acid; 99% acetic acid; saturated and half-saturated trichloroacetic acid, and alkali; 50% sodium hydroxide; 50% potassium hydroxide) burns in rats by immersing a hind leg in the solutions for various periods from 15 seconds to 1 minute. At a higher concentration of acids and bases, many animals receiving no decontamination died and developed severe burns. Neutralization with 5% sodium bicarbonate (acid burns) or 1% acetic acid (alkali burns) was compared with water decontamination either by holding the exposed limb under running tap water or by placing the rat in a large tank of water. In all cases, animals treated with neutralization developed less severe burns than untreated controls, while animals decontaminated with water developed less severe burns than those treated with neutralization (84). With exposure to 10% sulfuric acid, there were no burns even in untreated controls. However, with exposure to 25% sulfuric acid, all animals developed burns, regardless of decontamination modality.

Rats with skin exposure to 2N sodium hydroxide were washed with 500 mL of distilled water at a rate of 33 mL per minute beginning 1, 10, and 30 minutes after exposure (87). Subcutaneous tissue pH was measured at 1 minute intervals for up to 90 minutes following exposure. The peak tissue pH measurements were 9.97 for the 1-minute group, 10.57 for the 10-minute group, and 12.17 for the 30-minute group. The pH measurements for the groups with water washing begun at 10 and 30 minutes post exposure were not different from those seen in exposed controls that received no decontamination (87). Using the same rat model, a comparison was made of subcutaneous tissue pH between rats washed with 500 mL of distilled water and 500 mL of a 0.35 M sodium citrate solution as a neutralizing agent (88). As compared with the water decontamination group, subcutaneous tissue pH measurements were significantly higher in the 1-minute group and significantly lower in the 10- and 30-minute washing groups. Regardless of which decontamination measure was used, the upper layers of the skin became necrotic and deeper burns were observed in the groups with delayed (10 or 30 minutes) beginning of decontamination (88).

Brown et al. (1975) while studying the efficacy of polyethylene glycol (PEG) for decontamination of phenol and related compounds' exposure in rats, also compared PEG with water for decontamination of 45% sodium hydroxide and concentrated sulfuric acid exposures. Water decontamination was more efficacious than PEG, although burns of varying severity developed in all animals exposed to sulfuric acid and decontaminated with water, while burns of varying severity developed in 12/90 animals exposed to 45% sodium hydroxide and decontaminated with water (131).

Andrews et al. (89) challenged the dogma that skin burns caused by alkaline substances should be decontaminated with water and

that neutralization should not be attempted. In an experimental animal study of rats exposed to 2N sodium hydroxide, decontamination with 5% acetic acid was superior to water decontamination at attaining physiological pH, resulted in less severe tissue damage, and was associated with improved wound healing, thus supporting the idea that perhaps water decontamination is not the best intervention in alkaline chemical burns (89).

Human Case Reports

Older literature also described attempts at chemical neutralization following skin/eye chemical splash exposures. Terry (90) described the use of a 5% ammonium chloride solution for neutralization of sodium hydroxide followed by plain water washing which did not prevent significant burns. While no actual patient data were presented, this author stated that immediate 5% ammonium chloride solution irrigation "...in the great majority of cases prevented a burn; however, skin burns did occur when there was more than a 30–40 second delay before ammonium chloride irrigation was begun." For caustic soda (sodium hydroxide) eye splashes, this author recommended a five-minute irrigation with 5% ammonium chloride solution followed by a 55-minute irrigation with boric acid/saline solution, which "...has reduced the time of recovery of caustic soda burns of the eye from weeks to days" (90).

Carson (91) described three cases of extensive industrial burns. The first patient fell into a vat of a cresylic acid derivative (phenolic compound) and developed burns over 15% of the TBSA. Despite immediate removal of contaminated clothing and thorough water shower decontamination, he developed anuria, hypokalemia, and cardiac failure and died on the 10th day after the accident. A second patient slipped and inadvertently immersed his arm in a vat of chromic acid, developing an extensive burn of the entire limb despite immediate water washing. About 45 minutes later, the arm was again decontaminated with a phosphate buffer solution. Extensive excision and delayed skin grafting was required, necessitating 44 days in hospital. A third patient fell into a vat of hot (81ºC [~178º F]) nickel plating solution (nickel chloride, nickel sulfate, boric acid, and coumarin). Sodium bicarbonate was applied at the company infirmary and 40 minutes later a "buffer solution" compress was applied. Burns involved 40% of the TBSA. Hypotension, oliguria, and hyponatremia developed and the patient expired on the fifth day following the accident.

With relatively dilute sodium hydroxide (4%) oven cleaner aerosol exposure, full-thickness burns of the face requiring skin grafting may occur despite the lack of early pain (92). A patient with such an exposure did not present to the emergency department until two hours after aerosol exposure. The patient had wiped her face with a water-moistened cloth immediately after exposure, but did not irrigate her face with water. Continuous water irrigation in the emergency department did not significantly modify the pH of the patient's facial skin nor did it prevent the development of full-thickness burns requiring skin grafting (92).

A 20-year-old man fell into a caustic lime pit and developed an 85% TBSA burn (93). Treatment was delayed by more than 20 hours because of initial misdiagnosis and confusion over what the exposure actually involved, and he arrived at the burn center still covered with a thick, adherent layer of the lime. Decontamination in a water-filled Hubbard tank was only partially successful. Skin grafting was completed by 30 days after the injury, although some areas had to be secondarily debrided and regrafted with permanent wound closure obtained only after two months. Functional restoration was achieved at three months after injury (93).

O'Donoghue et al. (94) described three cases of caustic soda (sodium hydroxide) burns of the hand and feet. In two cases, the patients had immediate copious water decontamination; in the third case decontamination was not described. All three patients developed significant, deep necrotic burns requiring debridement and skin grafting. In two cases, recurrent necrosis occurred over 6 and 13 months.

A 36-year-old male was exposed to sodium hydroxide (pH 12–14) from a spilled barrel in a leather-processing factory (95). Contaminated clothing was immediately removed and the worker was washed with water continuously in a shower for approximately 20 minutes. On admission to the burn center, there were 52% TBSA burns present. Irrigation with water was done for a further two hours, and repeated six times with four-hour rest periods in between. Debridement and skin grafting were required over a 43-day hospitalization, following which pressure garments were worn for 18 months. No functional deficits were present at the conclusion of treatment.

Paulsen et al. (96) described two workers sprayed with liquid titanium tetrachloride while dismantling piping in a chemical plant. Titanium tetrachloride reacts with water releasing heat and hydrochloric acid, and should initially be dry-wiped from exposed areas before other types of decontamination are attempted. These two workers were perspiring heavily and the titanium tetrachloride reacted with perspiration, releasing hydrochloric acid. They were immediately dry-wiped with towels and then decontaminated with water in safety showers. On hospital admission, they had 18 and 20% TBSA partial-thickness injuries. One also had bilateral corneal burns. Both required debridement and skin grafting, and remained in the hospital for two weeks. Return to light duty was allowed after eight weeks (96).

Seven Saudi Arabian children had skin exposure to sulfuric acid when they tipped over a drum stored on the rooftop of their residential block (97). These children developed chemical burns of 3–60% TBSA. Contaminated clothing was not removed and water decontamination was not done until one-half hour after exposure. Following this, four children were treated and released and three children with 10, 15, and 60% TBSA burns were hospitalized. The child with 60% TBSA burns had 166 days of initial hospital admission, eight autografting and one homografting surgical procedures, and two further hospital admissions and surgical procedures for burn sequelae. In these seven children, one-half hour of water irrigation begun one-half hour after sulfuric acid skin exposure did not prevent burns and significant sequelae in one child.

Two workers had similar skin and inhalation exposures to liquid anhydrous ammonia and its vapor when a hose became disconnected from a river barge to a dock tank (98). One worker immediately left the area, showered with water, and removed contaminated clothing. He developed bilateral corneal burns, edematous and peeling lips, and hyperemia of the face and neck. After a day of hospital admission, rapid healing occurred. The second worker did not change contaminated clothing or shower immediately. On hospital arrival 90 minutes later, his face and lips were swollen, breathing was difficult, and endotracheal intubation was required to maintain a patent airway. There were 14% TBSA partial-thickness burns on the face, neck, chest, arms, hands, and thigh. Skin grafting was required and wound infection developed

in the thigh burn. Hospital admission was for 13 days. Although immediate water decontamination was associated with less severe burns in one of these two workers, it did not prevent burns from developing.

A 42-year-old man accidentally spilled hydrochloric acid on his arm. He had immediate water washing followed by irrigation with normal saline in an emergency clinic. Six hours after exposure, waterjet hydrosurgery was performed for debridement of the wounds, followed by daily dressing changes. Complete wound healing was noted by 19 days after exposure and no complications occurred (99). Immediate water decontamination did not prevent injury or the need for further treatment in this case.

A 22-year-old male electroplating worker went into a 3 m high electroplating tank to perform interior cleaning (100). The electroplating solution had not been completely drained away, and his legs were immersed in the solution, but he continued to work. After 10 minutes, pain developed and the worker immediately left the tank which was subsequently found to contain a chromic acid (hexavalent chromium) solution and he decontaminated his legs with copious amounts of water. However, both symptoms and sings of hexavalent chromium systemic poisoning and 15% TBSA skin injury developed. Hospitalization and intensive care treatment were required for 10 days and the patient was hospitalized for 33 days (100). In this case, immediate copious water decontamination did not prevent either skin injury or systemic toxicity.

A 21-year-old woman accidentally spilled glycidyl methacrylate on her hands and right foot during a laboratory experiment (101). Immediate hand washing with running water apparently prevented chemical hand injury. She did not wash her exposed foot until six hours later and developed second-degree burns on the foot (101). In this case, immediate water washing apparently prevented chemical hand injury, while delayed water washing did not prevent foot chemical skin injury.

Gasoline skin contact may cause significant chemical skin injuries with intact hairs (102). A patient involved in a roll-over automobile accident was trapped in the back seat for 1-½ hours. His pants were soaked with gasoline from a leaking fuel tank. Second-degree chemical injuries were observed in the exposed areas (10% TBSA). Delayed extensive water lavage of the exposed areas in the emergency department was carried out.

Case Series/Epidemiological Studies

In a study of 51 patients, to evaluate whether immediate water decontamination was adequate, patients were divided into groups and adequate treatment was defined as immediate dilution or neutralization therapy (103). The mortality in this study was 13%, most of the chemical burn patients were young men, and the injury was most often work related. The largest group of chemicals involved were alkalis (sodium and potassium hydroxides) followed by sulfuric acid, gasoline, anhydrous ammonia, white phosphorus, and hydrofluoric acid; four of the five deaths were work related. Although the group with immediate water decontamination had a generally shorter length of burn center admission and decreased mortality, this intervention did not prevent the development of burns or a 9.5% mortality (103).

Bromberg et al. (104) reviewed 273 chemical burns treated at two hospitals in New York during 1957–1963. Accidental exposures and deliberate assaults with caustic substances were approximately equally represented. Alkalis (potassium hydroxide, sodium hydroxide) were involved in 208/237 cases (75%) and resulted in more severe burns than did acids. The head and neck were most often involved and a high percentage of patients had concomitant corneal burns. A subset of 95 patients were further described, either receiving continuous water irrigation in a shower, brief water irrigation (30–60 minutes), continuous water soaks, or open treatment. Continuous water irrigation in a shower decreased the waiting period until skin grafting could be done to 22 days, as compared to 26–34 days with the other treatment modalities. It also decreased the average length of hospital treatment to 19 days as opposed to 23–39 days with the other modalities. The requirement for skin grafting was 20% less in the group receiving continuous water irrigation (104). Hydrotherapy, especially by immersion, has been associated with sepsis and alternative local wound care has been shown to decrease lethal infections complications (105).

Wolfort et al. (106) reported a series of 416 patients with lye injuries treated at two hospitals in Baltimore from 1952 to 1968. Of these, 42 had cutaneous lye burns involving 5–60% of the TBSA. Only nine injuries resulted from workplace accidents; the majority were deliberate assaults. The mean hospital admission time was 32 days and one fatality occurred but was attributed to an anesthetic accident rather than to the burn. Complications included tympanic membrane perforations (from lye running into the external auditory canal), parotid fistulas, a greater potential for keloid formation than seen with thermal burns, and the early appearance of Marjolin's malignant ulcers in the burn scar (seen at 3–9 years following lye burns and an average of 34 years following thermal burns). Treatment protocols at these two hospitals included early water decontamination followed by 12–24 hours of continuous water irrigation in a shower (106). Despite these interventions, all 42 patients developed burns requiring debridement and skin grafting.

Curreri et al. (107) described 111 patients with chemical burns treated at the U.S. Army Institute of Surgical Research over a 19-year period from 1950 through 1968. Of these, 96 patients had white phosphorus burns, 5 were burned with concentrated sulfuric acid, 3 with sulfur mustard, and 4 with other chemicals. There was a longer healing period for patients with chemical burns than for those with burns of other etiologies, although the mortality was less: 5.4% for patient with chemical burns as compared with an overall burn patient mortality of 10.5%. There was a high incidence of periorbital and ocular complications in the chemically burned patients.

Mozingo et al. (108) described 87 patients with chemical burns treated at the U.S. Army Institute of Surgical Research during a 17-year period between 1969 and 1985. The most common chemical involved was white phosphorus (49 cases). Other causative chemicals were acids (13 patients), alkalis (10 patients), organic solvents (5 patients), and a variety of other chemicals (15 patients). Patients with chemical burns from other than white phosphorus had shorter hospital admissions than other burn patients. Of the 38 patients with other than white phosphorus chemical burns, the average TBSA was 29.8%, the average third-degree TBSA burn was 17.8%, and the mortality rate was 26.3%. Three of these patients had associated eye injuries. Complications were noted in the non–white phosphorus chemical burn patients, including joint contracture (3 cases), pneumonia (2 cases), burn wound infection (2 cases), blindness (2 cases), myocardial infarction (2 cases), phenol toxicity (2 cases), pulmonary embolus (2 cases), brain

death (1 case), formate toxicity (1 case), nitrate toxicity (1 case), pancreatitis (1 case), and other (7 cases).

In patients with burn injuries treated at the U.S. Army Institute of Surgical Research from 1963 to 1968, 104 cases with ocular burn injuries were identified (109). Flame injury was the most common etiology, but chemical exposure was the cause in 27 patients.

In a study of a case series of cement-related injuries including a review of data from the U.S. National Burn Repository and the literature, 12 cases of cement burns (0.8%) were identified (110). These 12 alkali burns occurred in men aged 15–64 years. The TBSA involved was 0.25–10%, exposure times ranged from 1 to 6 hours, there was a treatment delay of one day to two weeks, and the time of hospitalization ranged from 2 to 14 days. Six of these patients required skin grafting. The authors note from their literature review that cement soaked into pants or spilled inside boots were common exposure routes and workers with job-related exposures were more likely to require skin grafting (110).

Hydrofluoric Acid Burns

Hydrofluoric acid (HF) is a relatively weak acid that penetrates deeply into the tissues resulting in severe burns. Hydrofluoric acid has widespread usage in industry (111).

High concentrations can also cause life-threatening systemic poisoning and fatalities (111–119). However, in one retrospective case series, over a 15-year period, HF burn size was most often small and did not result in electrolyte disturbances, significant morbidity, or death (121). Penetration of H+ and F− ions into the tissues causes the corrosive lesions; chelation of calcium leads to systemic hypocalcemia (122,123) and other serious electrolyte imbalances (hypomagnesemia and hyperkalemia may occur (124,125) leading to severe metabolic acidosis, cardiovascular shock, cardiac conduction abnormalities, and cardiac arrhythmias including Torsades des Points ventricular tachycardia and asystole Wu et al., (126); El Saadi et al., 1998). Immediate removal of HF-contaminated clothing and copious water washing has not prevented systemic absorption and significant systemic toxicity in some cases (126).

There are various methods for decontaminating and treated HF dermal burns (124,125,127–130,132,134). Concentrated (40–70%; anhydrous) HF rapidly produces painful lesions (135), requiring that decontamination and treatment be undertaken immediately. In spite of early water decontamination followed by repeated topical calcium gluconate inunction or subcutaneous injection, development of burns often cannot be prevented. The risk of systemic and sometimes fatal HF toxicity is greatest with concentrated HF splashes (116).

Dilute HF has sometimes been successfully decontaminated with water followed by topical application of calcium gluconate gel. The difficulty in such cases is the perception of the need to immediately undertake such measures in the absence of pain, which may be delayed in onset with more dilute solutions (135–138) and on occasion with more concentrated solutions, leading to possible delays in decontamination and treatment (139). The duration of the HF contact with the tissues may be prolonged in such situations. With dermal exposure to either dilute or concentrated HF, surgical debridement, excision, or even amputation of necrotic tissues may be required in certain cases (133,140–142).

Immediate water decontamination, especially with concentrated HF skin/eye splashes, is not sufficient to prevent tissue injury and systemic toxicity in many cases. In studies with 70% HF-exposed human skin explants ex vivo, as compared to untreated controls, water washing followed by topical application of calcium gluconate did not prevent tissue injury, but did decrease its severity and delay its onset (143).

DISCUSSION

Water decontamination has the following proposed mechanism of action (104): (*i*) dilution of the chemical agent; (*ii*) rinsing off the chemical agent; (iii) decreasing the rate of the chemical reaction; (iv) decreasing tissue metabolism and therefore the inflammatory reaction; (v) minimizing the hygroscopic effects of chemicals that produce them; and (vi) restoring normal skin pH in acid and alkali burns.

The ANSI/ISEA Z358.1–2009 Standard is a consensus standard for emergency water decontamination equipment for the skin and eyes (144). It specifies that emergency showers should deliver a pattern of flushing fluid 20 inches (50.8 cm) across with a flow rate of 20 U.S. gallons per minute (75.7 liters per minute) for 15 minutes and velocity sufficiently low so as not to be injurious to the user. For eyewash stations, a 15-minute uninterrupted water supply must be available and plumbed units should deliver no less than 0.4 U.S. gallons per minute (1.5 liters per minute) for 15 minutes with a velocity sufficiently low so as not to be injurious to the user (144). Emergency decontamination stations should be clearly marked and should take a chemical-exposed worker no longer than 10 seconds to reach.

For eye decontamination, it has been said that "The ideal flushing solution is a sterile, isotonic, preserved, physiologically balanced solution" (145). However, "At a minimum, flushing fluid would be clean and nontoxic," which would include potable water (water suitable for drinking) (145). There remains the problem of disposal of water used for skin/eye decontamination of chemical splashes, particularly whether a chemical can be discharged into the sanitary sewer system even in a very dilute concentration (146).

In a frequently cited review, Jelenko (147) noted that chemical agents do not "burn" in the classic sense of tissue destruction by heat. Rather, they act by coagulating protein through oxidation, reduction, salt formation, corrosion, protoplasmic poisoning, metabolic competition of inhibition, desiccation, or vesicant activity and its resultant ischemia.

Psychosocial consequences are often overlooked in patients with chemical burns, especially those involving the head, face, eyes, and neck. Rumsey et al. (149) found that the considerable portion of patients with disfiguring conditions, including burns, had psychosocial difficulties including increased anxiety levels, depression, social anxiety, social avoidance, and reduced life quality.

CONCLUSION

From this review, it is clear that while chemical skin/eye injuries represent a small portion of total burn injuries, their human and economic impact is significant. Although immediate water decontamination has generally been shown to decrease the severity of chemical skin/eye burns, it is also obvious that it does not always prevent such burns from developing, nor does it always

prevent the need for lost work time, hospitalization, burn center/unit admission, the requirement for surgical treatment, and sequelae. Significant sequelae and death can occur following chemical splashes, even when water decontamination is done on a timely basis.

Given a renewed interest in neutralization measures, decontamination solutions that are sterile, chelating, polyvalent (bind a wide variety of chemicals/chemical groups), amphoteric (bind opposed chemical groups such as acids/bases, oxidizers/reducing agents, etc.), nontoxic, hypertonic (to help prevent skin/corneal penetration), and water soluble (so that beneficial rinsing and diluting effects of water are not lost) should be critically evaluated by those concerned with initial decontamination of skin/eye chemical splashes. Comparative, blinded, controlled studies of various skin/eye decontamination solutions, including water, are needed.

ACKNOWLEDGMENT

Funding to prepare this review was provided by Laboratoire PREVOR, Valmondois, France.

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59 Irritant and allergic contact dermatitis treatment

Hongbo Zhai, Angela N. Anigbogu, and Howard I. Maibach

INTRODUCTION

The treatment of contact dermatitis (CD) including irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD) lies principally in the avoidance of the offending agent. Application of barrier creams (BCs) as well as wearing appropriate gloves and clothing is an important measure in the industry, to prevent or reduce ICD and ACD (1–3). In certain circumstances, avoidance of those annoying substances might not be practicable, and hence, treatment is rendered. Topical corticoids and other therapy approaches are "standard" therapy (4,5). This chapter reviews this topic and also briefly introduces some promising new therapies from the last decade, such as the immunomodulatory and UV light, and so on.

AVOIDANCE

Avoidance, that is, prophylaxis, is a key to "treatment." Protective gloves and clothing, as well as industrial hygiene and engineering, greatly improve management (2,3).

Moisturizers

Moisturizers are frequently used to improve "dry" skin, and daily use may modify the physical and chemical nature of the skin's surface, so as to smoothen, soften, and make it more pliable (6,7).

Moisturizers often contain humectants of low molecular weight and lipids. They are absorbed into the stratum corneum and thereby attract water and increase hydration (8). Lipids, for instance, petrolatum, beeswax, lanolin, and the various oils in moisturizers are incorporated into formulations on the basis of their technical and sensory properties, rather than on their possible epidermal impact (8,9). They may also penetrate the living epidermis, be metabolized, and significantly modify the endogenous epidermal lipids (10). A single application of a moisturizer does not cause long-lasting effects, expressed as skin capacitance and conductance (11,12), whereas, repeated applications of a moisturizer twice daily, for one week, produces a significant increase in skin conductance for at least one week post-treatment (13).

Urea, a physiological nonallergenic substance (14,15), can reversibly decrease the turnover of epidermal cells (16), and may also enhance the penetration of other substances into the skin (14,17,18). Other effects include binding water in the horny layer, antipruritic, and reducing irritant dermatitis (14,15,19,20). Zhai and Maibach (6) reviewed the subject of moisturizers in preventing ICD. Extensive data on the physiology, pharmacology, and toxicology of moisturizers can be found in Loden and Maibach (21). Kütting et al. (22) provide product-specific data from a controlled efficacy study in the metal working industry.

Barrier Creams

BC are designed to prevent or reduce the penetration and absorption of hazardous materials, preventing skin lesions or other toxic effects from dermal exposure (23–28). Their efficacy has been investigated by in vitro and in vivo studies (24,26,27,29,30). However, their actual benefit remains *sub judice* in clinical trials (24–26,28,29,31–37). Inappropriate BC application may exacerbate, rather than ameliorate, the problem (24,25,28,31–34). In practice, BCs are usually recommended only for low-grade irritants (water, detergents, organic solvents, cutting oils) (28,29,32). BCs are also used to protect the face and neck against chemical and resinous dust and vapors (38).

Reasons, mechanisms, application methods, and general topics of BC can be found in Zhai and Maibach (1), as well as related chapters of this book.

Protective Gloves and Clothing

Gloves may provide certain protective effects against corrosive agents (acids, alkalis, etc.) (29,39,40). Protective clothing as well as other personal devices also play a critical role (41,42). Note that protective clothing may trap moisture and occlude potentially damaging substances next to the skin for prolonged periods and increase the likelihood of dermatitis developing (41,42). The first line of defense against hand dermatitis is to wear gloves, but in many professions it is impossible to wear gloves, because of the loss of dexterity. In some instances, an alternative will be to utilize BC. Note that many gloves do not resist the penetration of low molecular weight chemicals. Some allergens are soluble in rubber gloves, and may penetrate the gloves and produce severe dermatitis (3,29,41,43–45). Recently, allergy to rubber latex has become a growing problem (3,29,43–45), and workers can develop the contact urticaria syndrome, including generalized urticaria, conjunctivitis, rhinitis, and asthma (29,46). Updated document details in this area can be found elsewhere (3,45).

TREATMENT

Corticoids

Hydrocortisone, which became available in the 1950s, was shown to be efficacious in eczematous dermatoses (47). The next major

advance in topical corticoid therapy came with the introduction of triamcinolone acetonide, followed shortly after by flucinolone acetonide. The early 1970s saw the introduction of the 21-acetate derivative of flucinolone acetonide, with more biological activity than the others. Since the late 1970s, many potent topically active glucocorticoids have been introduced, including desoximetasone, clobetasol propionate, and betamethasone-17-dipropionate.

Mechanism of Action

Corticoids being lipophilic in nature permeate the skin by passive diffusion. Following the penetration of the cell membrane, corticoids bind with specific cytoplasmic receptors. These receptors have been demonstrated in all target tissues including the skin (48).

As inflammation is the endpoint of the immune response, the anti-inflammatory and immunosuppressive effects of corticoids may overlap (49–54).

The mechanisms by which topical corticoids cause vasoconstriction remain unclear (55–58).

The effects of topical application of corticoids on human mast cells have been examined (59). Two potent corticoids, clobetasol-17-propionate and fluocinonide, produced >85% decrease in histamine content over six weeks of treatment. The first signs of cells containing sparse amounts of mast-cell granules were apparent 14 days poststeroid treatment. By three months, the histamine levels returned to normal. This study suggested a possible treatment for one human mast-cell disease, urticaria pigmentosa, and a possible additional mechanism of action of corticoids. Maibach and Surber (4) and Korting and Maibach (60) provide additional details.

Percutaneous Penetration

Following topical application, corticoids penetrate the stratum corneum and are absorbed into the epidermis. The efficacy and toxicity are related to corticoid penetration. Corticoids may act on the epidermis, the dermis, or both.

Topical corticoids applied to diseased skin will be absorbed into the systemic circulation. When administration is chronic or when large areas of skin are involved, the absorption may be sufficient to cause systemic effects, including cushinoid changes and adrenocortical suppression.

Topical corticoids are minimally absorbed from healthy skin. On the forearm, approximately 1% of the applied dose of hydrocortisone penetrates the skin (61). Other corticoids, for which data exist, are not necessarily absorbed to a greater degree than hydrocortisone (62), suggesting that they may owe their increased efficacy to their potency rather than to enhanced penetration.

Clinical Formulations and Potency of Corticoids

Corticoids form a vast range of compounds and formulations, with varying effects. Table 59.1 groups topical corticoids according to relative potency, largely based on the vasoconstrictor assay (63). The formulations in each group are only roughly equipotent. It is concluded that the greater the potency, the greater the therapeutic efficacy and likelihood, therefore, of more adverse effects.

Superpotent formulations include clobetasol propionate, optimized betamethasone diproprionate, and diflorasone, and must be used with caution; they have the potential for significant topical and systemic side effects, far in excess of other currently used formulations.

TABLE 59.1
A Partial List of Topical Corticoids Available in the United States Ranked According to Their Potencies

Drug	Potency (%)
Lowest Potency	
Hydrocortisone	0.25–2.5
Methylprednisolone acetate	0.25
Dexamethasone[a]	0.04
Dexamethasone[a]	0.1
Methylprednisolone acetate	1.0
Prednisolone	0.5
Betamethasone[a]	0.2
Low Potency	
Fluocinolone acetonide[a]	0.01
Betamethasone valerate[a]	0.01
Flurometholone[a]	0.025
Aclometasone dipropionate	0.05
Triamcinolone acetonide[a]	0.025
Clocortolone pivalate[a]	0.1
Flumethasone pivalate[a]	0.03
Intermediate Potency	
Hydrocortisone valerate	0.2
Mometasone furoate	0.1
Hydrocortisone butyrate	0.1
Betamethasone benzoate[a]	0.025
Flurandrenolide[a]	0.025
Betamethasone valerate[a]	0.1
Desonide	0.05
Halcinonide[a]	0.025
Desoximetasone[a]	0.05
Flurandrenolide[a]	0.05
Triamcinolone acetonide[a]	0.1
Fluocinolone acetonide[a]	0.025
High Potency	
Betamethasone dipropionate[a]	0.05
Amcinonide[a]	0.1
Desoximetasone[a]	0.25
Triamcinolone acetonide[a]	0.5
Fluocinolone acetonide[a]	0.2
Diflorasone diacetate[a]	0.05
Halcinonide[a]	0.1
Fluocinonide[a]	0.05
Highest Potency	
Betamethasone dipropionate[a] in optimized vehicle	0.05
Diflorasone diacetate[a] in optimized vehicle	0.05
Clobetasol propionate[a]	0.05

[a]Fluorinated steroids.

Vehicles

The potency of topical corticoids can be further enhanced by stimulating percutaneous absorption. One such way of optimizing absorption is by altering the formulation vehicle (63). Ointment bases tend to give greater activity to the corticoid than do cream or lotion vehicles (63).

Adverse Effects

All absorbable corticoids possess the ability to produce adrenal suppression (64,65). The degree of suppression is related to the potency. Fortunately, plasma cortisol usually returns to normal within three days when the superpotents are discontinued—at least in short-time application studies (66).

Certain factors such as application to large surface areas, occlusion, inflamed skin, and higher concentrations, may increase the penetration, and therefore, the tendency to suppression. Of concern, in children, is growth retardation, associated with excessive and prolonged use of topical corticoids (67–70).

Dosage and Administration

Most physicians prescribe topical corticoids with little or no thought as to the number of milligrams of material per surface area of skin. There is a dosage–response relationship, with increasing efficacy, closely following an increased dosage. It is therefore important to make an estimate of the quantity a patient will require in any given condition. Fortunately, most manufacturers provide a standard or regular concentration yielding the desired therapeutic result for most patients. For instance, triamcinolone acetonide is available in 0.025%, 0.1%, and 0.5% formulations. Many patients with corticoid-responsive dermatoses need only the 0.025% formulations.

The standard trade concentrations suffice for most patients. In the more resistant diseases, higher concentrations should be considered. For instance, approximately 1% of a 0.25% hydrocortisone solution is absorbed from the forearm. Increasing the amount applied per unit area of skin 10-fold, increases the amount absorbed four times (71).

Regional differences in response are partially based on the differences in penetration of skin in various areas. Thus, areas with increased permeability, such as the scrotum, eyelids, ears, scalp, and face, respond far better to topical corticoids than areas such as the dorsa of the hands, extensor surfaces of knees and elbows, and the palms and soles (72).

Occlusion

Occlusion of 96 hours with an impermeable film, such as a plastic wrap, constitutes the most effective method of enhancing penetration, yielding approximately a 10-fold increase (73). Specifically, with occlusion, penetration of hydrocortisone on the forearm increases from 1% of the applied dose to 10%. There are, however, obvious problems associated with occlusion therapy—the plastics are sometimes uncomfortable, warm, and troublesome to use. Side effects encountered with occlusion include miliaria, bacterial, and candidal infection.

Occlusion has the added advantage of keeping the drug on the skin by preventing rubbing off onto the clothing. We do not have data delineating the effect of the duration of occlusion on percutaneous penetration with topical corticoids.

Frequency of Application

Previously, patients applied topical corticoids three to four times daily. Studies on the percutaneous absorption of hydrocortisone failed to reveal a significant increase in absorption when applied on a repetitive basis compared to a single dose (74). Clinical trials of various corticoids suggested that less-frequent applications were equally effective (75). In view of the relatively slow process of corticoid absorption, a phenomenon referred to as the "reservoir effect," (76) there may not be any advantage in frequent applications.

Acute tolerance (tachyphylaxis) to vasoconstriction and antimitotic effects of suppression of epidermal DNA synthesis by topical corticoids have been demonstrated (77,78). This suggests that the resistance clinically observed after prolonged use might be prevented by less intensive therapy, such as daily application, with short resting periods between treatment courses (79,80). Another study examining corticoid tachyphylaxis used fluocinolone acetonide under occlusion to the forearm and induced wheal and flare to histamine with the prick technique (81). By the eighth day, the wheal was nonexistent, adding now a third tachyphylaxis phenomenon.

Anatomic Variation

Large regional variations in the percutaneous absorption of compounds are determined by factors including hair follicle density, thickness of the stratum corneum, and vasculature of the region (82). This suggests that for areas of higher penetrability such as the face, scalp, scrotum, axilla, and the groin, smaller doses are required and occlusion is not needed (71).

Little quantitative information is available on how much penetration is increased in the diseased skin (83). In initial studies, it was noted that skin with only minimally involved atopic dermatitis allowed for a several-fold increase in penetration; psoriatic plaques had no significant increase, whereas, exfoliative psoriatic skin had little barrier to penetration.

CONTROLLED TOPICAL EFFICACY STUDIES: IRRITANT AND ALLERGIC CONTACT DERMATITIS

Irritant Dermatitis

Most physicians employ topical corticoids in irritant dermatitis; however, several controlled studies in experimental irritant dermatitis to sodium lauryl sulfate (SLS) show either no, or a negative (84), or a minimal effect (85).

Allergic Contact Dermatitis

Several studies document some degree of efficacy when high potency corticoids are applied after the acute phase (86). Considering the massive amounts prescribed, the data are limited—possibly because this has long been the standard of care.

Immunosuppressives

Cyclosporin and azathioprine are used in unusual instances. See Menné and Maibach (44) for details.

Calcineurin Inhibitors

Controlled studies with calcineurin Inhibitors (tacrolimus and pimecrolimus) suggest some efficacy (87,88). Ott et al. (87) investigated tacrolimus-dependent immunomodulation on gene expression alterations in human antigen-presenting cells, which are stimulated with small-molecular-weight contact allergens, and

demonstrated that calcineurin inhibitor tacrolimus has modulatory effects during the sensitization phase of ACD. Engel et al. (88) tested the anti-inflammatory effect of pimecrolimus cream after damage of the skin barrier by SLS in a randomized, placebo-controlled, observer-blinded study. In their study, 3% SLS was applied, under occlusion, on the back of 36 healthy volunteers, for 24 hours. Subsequently, the test areas were treated for 24 hours with pimecrolimus cream, 1% hydrocortisone in a hydrophilic ointment, and the vehicle alone, over three consecutive days. One control area remained untreated. The erythema index and the transepidermal water loss (TEWL) served as readout parameters to assess the SLS-induced skin irritation. Results showed that the pimecrolimus cream and 1% hydrocortisone cream significantly reduced the SLS-induced erythema. However, until their black box warnings (for malignancy) are clarified, they remain a secondary treatment of choice (89)

UV LIGHT

Most patients with ICD and ACD may be controlled by topical therapy and protective measures. However, some cases cannot be controlled either topically or by acceptable doses of systemic corticosteriods. In these situations, UV treatment should be considered. Christensen (90) and Menné and Maibach (44) provide the details for the UV treatment regime.

GRENZ RAY

Grenz ray may act as an adjunct topical therapy in some chronic cases. In addition, it is extremely suitable if one considers the sparing effect of Grenz radiation on hair roots, and sebaceous and sweat glands. Details are provided by Lindelöf (91) and Menné and Maibach (44). Warner and Cruz (92) provide overviews of the evidence for and against these interventions.

CONCLUSION

Taken together, most patients respond to the current therapy; yet, considering the frequency of these conditions, much evidence-based data and refinement await investigation. In 2010, Kütting et al. (22) have concluded that the skin protection regimen seems to provide effective prevention of occupational skin diseases, from a prospective randomized controlled trial over a follow-up period of one year. Cohen and Heidary (93) review the treatment options for CD and provide more insights on this arena.

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60 Anti-irritants: Myth or reality? An overview

Kaley A. Myer and Howard I. Maibach

INTRODUCTION

Irritant contact dermatitis (ICD), a condition with multifactorial causes, results from acute and chronic exposure to chemicals found in cosmetics, personal care products, drugs, and during occupational exposure. Prognosis for chronic ICD may be poor; the disease results in lost work and causes significant distress. Thus, we sought to identify substances with anti-irritant potential in hopes of improving our understanding and better serving future patients. The concept of anti-irritants is prevalent but these substances are often considered part of a marketing ploy rather than valid methods of reducing ICD. Our overview attempts to add the available science to this concept.

METHODS

We performed a literature search using PubMed and EMBASE and a hand search of the library at UCSF in an attempt to investigate products that can be considered anti-irritants in either prevention or treatment.

Study Selection

Emphasis was placed on studies that included quantitative and qualitative results and that followed evidence-based and dermatological guidelines. We defined an anti-irritant as a moiety that either inhibits (prevents) or treats ICD. For the purposes of this review we focused on clinical markers of irritation, that is, edema, erythema, vesiculation, and diminished barrier function, as these are more readily and objectively assessed via visual scoring criteria, transepidermal water loss (TEWL) measurements, and erythema indices.

RESULTS

Glycerol

Glycerol has long been used as an anti-irritant. Two studies looked at glycerol's efficacy. Andersen showed in a randomized, double-blinded trial that the glycerol reduced TEWL and irritation due to sodium lauryl sulfate (SLS)/nonanoic acid (NON) at low concentration (1). However, Clemmensen failed to show in a randomized, placebo-controlled double-blinded study that it was more effective than the untreated and vehicle controls (2). It may be that Clemmensen's study did not have sufficient power to determine significance.

Retinoids

Two studies attempted to reduce the irritation potential of retinoid-based products. Alirezai et al. tested the hypothesis that Avene® medical spring water applied *ad libitum* to acne-affected/retinoid-treated areas would reduce the erythema, scaling, sting, and burn associated with retinoid compounds (5). This was a controlled, open-labeled, randomized study in which patients over the age of 12 years with moderate to severe acne were given either Retin-A® (Johnson and Johnson Laboratories, Raritan, New Jersey, USA) treatment alone (34 patients) or retinoic acid and mineral water (35 patients) to spray *ad libitum* (at least four times daily). There was no placebo-controlled group. Patients were assessed at the end of one and four weeks. After 28 days, patients treated with retinoic acid and mineral water showed reduced scaling (46% of patients treated with retinoic acid and mineral water complained of scaling, compared with 79% of patients treated with retinoic acid alone; groups were similar in size.) Overall tolerance of retinoic acid treatment improved with mineral water (37% vs 12% of patients rated their experience as "very good"). The results suggested that mineral water did not alter the therapeutic action of retinoic acid. Rather, those patients treated with both retinoic acid and mineral water showed a slight reduction in their overall acne; whether this was due to the combined therapy or increased compliance secondary to decreased unpleasant side effects was not elucidated. One question raised, but not answered, however, is whether the decrease in irritation shown was due to a simple dilution effect or to actual chemical properties of the mineral water itself.

Kim et al. used retinoid-induced irritation to investigate the cytokine mediators involved. Application of retinoids to human epidermal cells increased mRNA expression of the cytokines, monocyte chemoattractant protein (MCP-1), and interleukin 8 (IL-8). They then tested various potential anti-irritant substances for their efficacy in inhibiting these cytokines within in vitro human fibroblasts and conducted human in vivo patch tests (Draize skin irritation test) to test these same substances against retinol-induced irritation. SC-glucan (a soluble biopolymer produced by *Schizophyllum commune*) was effective at reducing retinol-induced irritation in human and rabbit models; in vitro it showed a mild inhibition (10.8%) of MCP-1 and IL-8 (4).

Surfactants

Schliemann-Willers studied the effects of natural fats against sodium laurel sulfate (SLS) induced irritation in a randomized study of 20 healthy volunteers tested with a repetitive irritation test. Rapeseed and palm fats had a significant beneficial effect against SLS-induced irritation: rapeseed decreased erythema by 2.7 (visual score); palm fats decreased erythema by 2.5, and TEWL by 25.1% compared with control. Both substances, though, offered weaker protection than Eucerin (Beiersdorf, Hamburg, Germany) and petrolatum (5).

Han et al. studied the anti-irritant capacity of aloe vera gel by combining it with varying strengths of SLS in 15 volunteers. SLS was dissolved in distilled water to 10, 20, 50, and 100%, and then the SLS solution was mixed with each of the aloe solutions in a 1:1 ratio. This mixture was randomly applied to volar forearms and left occluded under Finn Chambers® (*Epitest*, Helsinki, Finland) and filter paper for 24 hours. Both TEWL and erythema index (E-index) decreased significantly with the 100% aloe vera gel-SLS mixture. Note that aloe vera when tested alone also significantly decreased TEWL and E-index over the three weeks the patients were followed. These findings bear particular clinical relevance in that aloe vera seems to have long-term protective effects on the skin when used alone and when combined with known irritant products; no pretreatment was necessary to have the desired effect (6). However, like the retinoid-mineral water study, this study is also limited by the problem of possible dilution effect. Standard deviations or normal distributions were not shown; it is difficult to know whether the reduced irritation was due to active ingredients in the aloe vera itself or due to dilution and subsequent reduced percutaneous penetration of the SLS.

Andersen, using a forearm wash test, studied the effects of canola oil as traditional treatment for SLS/NON-induced irritant dermatitis and found that is was not more effective than controls (untreated site and vehicle) (7). One possible explanation for the lack of significant differences between treatments is that the washings used in the experimental design did not allow for friction to exacerbate irritation and thus differences in treatment accounted for little of the variations in responses.

Perfluoro-polyethers

Schliemann-Willers et al. tested 5% solutions of perfluoropolyethers (PFP) phosphate gel against four standard irritants commonly found in occupational sites: 5% SLS, 0.5% NaOH, 20% lactic acid (all hydrophilic), and undiluted toluene (hydrophobic). Volunteers were pretreated with PFP and then 30 minutes later the irritant solutions were applied in this randomized, placebo-controlled double-blind study. After two weeks they noted a significant dose-related prevention of the experimentally induced occupational ICD (9).

IMMUNE MEDIATORS

Phosphodiesterase Inhibitors

Kcharekova et al. studied the anti-inflammatory/anti-irritant effects of cipamfylline in 10 subjects. They compared the anti-inflammatory effects of betamethasone with those of cipamfylline (PDE-4 inhibitor) and placebo in this randomized blind study and found that betamethasone alone showed statistically significant reduction in TEWL (approximately 8 $g/m^2/h$ and E-index) (10).

Goyarts et al. showed that topical cyclic adenosine monophosphate PDE inhibitors have a moderate anti-inflammatory effect against Balsam of Peru. One major problem with this study is the fact that the irritation induced by Balsam of Peru is not typical irritation, but rather is nonimmunologic contact urticaria (11).

Corticosteroids

Ramsey and colleagues showed a significant decrease in TEWL (10%) and erythema with betamethasone (applied twice daily for 7 days) when compared with the SLS-irritated skin left untreated in 16 volunteers. Similarly, the Berardesca study showed significant decreases in TEWL with the application of methylprednisolone. Neither study, however, listed mass per unit area of corticoid applied (12,13). More recently, Clemmensen showed clobetasol improved SLS/NON-induced irritation in a dose-dependent manner, but these results were not statistically significant when compared with untreated and vehicle controls (2).

Sulfur Mustard

Sulfur mustard causes immediate blistering and affects primarily the skin, eyes, and respiratory system, with cutaneous manifestations occurring in two stages (early and late) that often require prolonged hospitalization and healing times. Two sulfur mustard studies show significant promise in the development of a product useful for lessening acute sulfur mustard-induced irritation (14,15). Dachir et al. effectively used a topical steroid/nonsteroidal anti-inflammatory drug (NSAID) combination to reduce edema, blistering, and epithelial damage (14). Similarly, Yourick and colleagues diminished erythema with combinations of niacinamide and promethazine and niacinamide, promethazine, and indomethacin, although serious skin inflammation and injury still occurred (15). Currently, however, these studies bear uncertain clinical significance, as experiments have not yet been performed in human volunteers.

There have been, however, experiments on human volunteers looking at control for chronic symptoms post sulfur mustard exposure. Panahli compared cutaneous application of pimecrolimus and doxepin to betamethasone: all three substances relieved the chronic pruritus and burning sensation and improved the quality of life for the patients. However, it is difficult to determine the significance of these results as the trials did not contain a placebo controlled group (16,17).

Natural Products

Levin described the use of diluted homeopathic gels (made from *Urtica urens, Apis mellifica, Belladonna, Pulsatilla*) as remedies for the inflammation and vasodilatory erythema caused by methyl nicotinate. Also reviewed is the use of two oils, borage and lavender, for the inhibition of atopic symptoms (pruritus, erythema, vesiculation, and oozing) (18). No mention is made as to how these substances affect similar ICD symptoms. The importance of these findings cannot be completely understood; however, as further examination of drug vehicles, potency and side-effect profiles must be performed to truly determine the extent of clinical relevance concerning such “natural therapies.”

Fuchs investigated the anti-irritant properties of rosemary, undyed and dyed marigold and faradol-ester-enriched extracts using both adjuvant and traditional treatments. They found with each of these products that erythema scores were improved only with the adjuvant treatment and TEWL scores did not improve with any of the treatments. This may be due to their antioxidant properties that prevent damage by the irritants but is less effective after the skin has sustained damage (19).

Glycolic Acid

Perricone and DiNardo studied the anti-inflammatory effect of topical glycolic acid on skin previously irradiated with the minimum erythema dose of UVB. When UVB-burned skin was treated with glycolic acid for 7 days straight, a 16% reduction in irritation could be observed (20).

Strontium Salts

Hahn defined an ideal treatment for sensory irritation, namely, the subjective complaints of burning, itching, tingling, and stinging (21). The information remains relevant for two reasons: (1) his research identified that strontium salts, whether mixed with nitrate or chloride, could act as anti-irritants when used as an adjuvant to aluminum or zirconium applications, especially in terms of reducing erythema (2); he showed multiple ways in which strontium effectively reduces sensory irritation—perhaps of less interest to the dermatologist—but surely an important patient concern. We hesitate to classify this as an example of "anti-irritation," as the physical injury from the irritant presumably still exists, and a topical anesthetic could be equally effective in blunting the sensory effects. Still, his work may represent a new avenue to follow in the search for effective anti-irritant substances.

Topical Nonsteroidal Anti-Inflammatory Agents

Topical NSAIDS, such as diclofenac and naproxen sodium are widely used in Europe and Asia as topical anti-inflammatory agents. Their efficacy has been well established for musculoskeletal systems. Although many irritant reactions include dermal inflammation, their value as anti-irritants for skin will require further investigation, and they were not included herein as there exist few evidence-based conclusions regarding their dermatologic use. The major exception consists of a body of experiments documenting that NSAIDs inhibit UVB erythema when used prophylactically (6). Likewise, experiments have been done proving their ability to inhibit nonimmunologic contact dermatitis, but that mechanism does not apply to true ICD (6). Finally, it bears mentioning that such products require further testing as they may themselves cause irritation in sensitized patients (21).

Calcineurin Inhibitors

The experimental data for tacrolimus and pimecrolimus relate solely to treatment rather than prevention, and is generally specific to psoriasis and atopic dermatitis. Off-label use of both drugs has been recommended for treatment of allergic contact dermatitis (23). However, a recent study looking at traditional treatment of NON- and SLS-induced dermatitis with tacrolimus showed no increased efficacy when compared with untreated sites and vehicle controls (2).

CONCLUSIONS

The data on anti-irritants are incomplete, and the studies herein presented (Table 60.1) prove that much remains to be done to properly identify substances that can be defined as true anti-irritants. There are, however, significant problems with these studies, as mentioned throughout. The mechanism of action of different anti-irritants is inherently useful information in terms of refining future technologies. Some mechanisms are readily understood, for example, barriers that minimize penetration: others are not readily comprehended in spite of decades of study. Petrolatum, for example, would fit this characterization. From our investigations there appear to be at least two potential mechanisms to inhibiting irritation: (1) inhibition of percutaneous penetration into the epidermis and dermis (24) and (2) altering the biochemistry and metabolism of the irritant compound as it is applied to the skin. Still, what is lacking is a well-controlled randomized and double-blinded study that has sufficient power, adequate number of controls, and that sufficiently tests both sensitivity and specificity of the anti-irritant to the irritant substance. We propose that the ideal experiment would test the sensitivity of the anti-irritant by having two controls: one tested against the anti-irritant and the irritant mixed together and one testing the anti-irritant as traditional treatment (applied to the skin after irritation developed). The ideal anti-irritants may in fact exist; further investigation, however, remains to be done.

TABLE 60.1
Anti-irritants and their potential benefits

Product	Potential Benefit	Treatment Type	Study Design	Comments	References
Glycerol	Proposed treatment of surfactant- induced irritant ICD via improved barrier function	Immediately after irritant application	Randomized, double-blinded	Reduced irritation after exposure to SLS/NON, reduced TEWL at low concentrations	(1,25)
Urea	Humectant	Traditional and pretreatment	Randomized, placebo-controlled, double-blinded	Pretreatment showed no significant difference in TEWL from placebo. Long-term treatment improved ICD from SLS/NON	(26)
Cold	Decreased inflammation and improved barrier function	Pretreatment and adjuvant	Randomized, single-blinded	Decreased irritation, measured by TEWL, skin color reflectance, and visual scoring	(27)

(Continued)

TABLE 60.1
Anti-irritants and their potential benefits *(Continued)*

Product	Potential Benefit	Treatment Type	Study Design	Comments	References
Tacrolimus	Decrease irritation after SLS/NON	Traditional (day 7 after irritation established after irritant washings)	Randomized, controlled, double-blinded	Not more effective than controls at reducing irritation measured by TEWL and ESCD guidelines	(2)
Clobetasol	Decrease irritation after SLS/NON	Traditional (day 7 after irritation established after irritant washings)	Randomized, controlled, double-blinded	Not more effective than controls at reducing irritation measured by TEWL and ESCD guidelines	(2)
Canola Oil	Improve barrier function	Traditional (1 day after induction of irritation, BID to follow)	Randomized, double-blinded	No improvement in SDS/NON- induced irritation acutely or chronically	(7,8)
Nifedipine	Anti-inflammatory	Traditional (1 day after induction of irritation, BID to follow)	Randomized, double-blinded	No improvement in SDS/NON- induced irritation acutely or chronically	(7,8)
a-bisabolol	Inhibits arachidonic acid pathway	Traditional (1 day after induction of irritation, BID to follow)	Randomized, double-blinded	No improvement in SDS/NON- induced irritation acutely or chronically	(7,8)
Sao Pedro do Sol mineral water	Ions improved barrier recovery, enhance natural moisturizing factor	Traditional (1 day post exposure)	Randomized	Decreased TEWL compared with purified water with SLS	(28)
Leopoldine Spa Water	Anti-inflammatory	Adjuvant	Unknown	Moderately decreased irritation scores (redness measured by chromometer) compared with double-distilled water with exposure to SLS	(29)
Seawater	Inhibit keratinocyte inflammatory cytokine production	Immediately after irritant application	Randomized	Sea water, NaCl, KCl decreased TEWL compared with deionized water	(30)
Avene Medical Water	Reduces scaling associated with retinoids	Adjuvant and traditional treatment	Controlled, open-labeled, randomized. 34 patients used retinoic acid alone for 28 days, 35 patients combined retinoic acid and mineral water. Patients instructed to apply mineral water *ad libitum*	No significant reduction of erythema, burn, or itch	(3)
Rosemary, undyed/dyed marigold, and faradol-ester-enriched extracts	Reported to neutralizes free radicals	Adjuvant and traditional	Randomized, placebo-controlled, single- blinded	Improves erythema only with concurrent treatment, no improvement with traditional treatment, no improvement in TEWL	(19)
Xylitol	Water retention in stratum corneum	Adjuvant	Unknown	At high concentration (15%), prevents elevation of TEWL. No difference between LS+xylitol and SLS alone	(31)
Mannitol	Water retention in stratum corneum	Adjuvant	Unknown	At high concentrations (18%), lower TEWL with SLS+ mannitol than SLS alone	(31)
Taurine	Antioxidant, anti-inflammatory	Adjuvant	Unknown	High concentration (8.4%) had lower TEWL with SLS+taurine than SLS alone	(31)
Glycine	Skin repair, wound healing	Adjuvant	Unknown	No effect on TEWL	(31)

(Continued)

TABLE 60.1
Anti-irritants and their potential benefits *(Continued)*

Product	Potential Benefit	Treatment Type	Study Design	Comments	References
SC-glucan	Effective inhibition of cytokine-mediated inflammatory response to retinoids; decreased erythema and edema	Adjuvant and pretreatment	Controlled, open-labeled	Moderate in vitro results tested against human dermal fibroblasts. Good in vivo results in both rabbit and human patch tests	(4)
Homeopathic gels (Utica urens, Apis mellifica, Belladonna, Pulsatilla)	Decreased inflammation caused by methyl nicotinate	Pretreatment	Unknown	Methyl nicotinate: nonimmunologic contact urticaria is a primarily pharmacologic effect; low clinical significance for irritant dermatitis	(32)
Borage oil	Improved pruritus, erythema, vesiculation, and oozing in atopic patients	Traditional treatment	Unknown	High in gamma-linoleic acid, presumably the active ingredient	(33)
Aloe vera gel	Improved skin barrier function (decreased TEWL) and decreased erythema caused by topical surfactant (SLS)	Adjuvant therapy	Placebo-controlled, randomized	Dose-dependent results: 100% aloe vera showed most significant results. Composed of multiple ingredients: actives not entirely known	(6)
Cipamfylline (selective phosphodiesterase-4 inhibitor)	Proposed treatment of surfactant-induced (SDS) ICD via cytokine inhibition	Traditional therapy	Controlled, blind, randomized	No significant reduction in erythema or TEWL compared with placebo or betamethasone	(10)
Strontium nitrate/chloride	Significantly reduced erythema due to aluminum/zirconium salt solution	Adjuvant therapy	Double-blind vehicle-controlled, randomized	Nitrate and chloride showed similar results. Also very effective against sensory irritation	(21)
Cyclic adenosine monophosphate phosphodiesterase inhibitors	Reduced irritation due to 8% Balsam of Peru	Traditional therapy	Unknown	Topical Balsam of Peru causes nonimmunologic urticaria, not ICD; results may be coincidental	(11)
Corticosteroid (betamethasone-17-valarate, methylprednisolone aceponate)	Decreased TEWL after SLS-induced irritation	Traditional therapy	Randomized, controlled, open	Anti-irritant efficacy noted at the end of treatment trial	(12)
Perfluoro-polyethers (oil in water emulsion)	Decreased TEWL and erythema due to SLS, NaOH, and 20% lactic acid	Pretreatment and traditional therapy	Randomized, double-blinded, controlled	Dose-dependent results with 5% PFPs showing optimal effect. Applicable to ICD conditions caused by occupational surfactant materials	(5)
Glycolic acid (oil in water vehicle)	Reduced irradiation-induced erythema	Traditional therapy	Controlled, open	Area treated for 7 days	(20)
Natural vegetable fats (rapeseed and palm)	Decreased irritation due to SLS	Pretreatment	Randomized, controlled	Reduced irritation less that Eucerin or petrolatum	(9)
Lipophilic extracts of Isatis tinctoria	Decreased erythema and TEWL due to SLS	Traditional treatment	Controlled, open, randomized	Significant activity against relevant targets of inflammation	(34)
Pimecrolimus	Decrease chronic pruritus from sulfur mustard	Traditional (17–21 yrs post exposure)	Investigator-blinded, randomized, controlled	Similar to Betamethasone in decreasing pruritus, burning sensation, and dryness but did not show the same improvement in scaling. No placebo group.	(17)

(Continued)

TABLE 60.1
Anti-irritants and their potential benefits *(Continued)*

Product	Potential Benefit	Treatment Type	Study Design	Comments	References
Doxepin	Decrease chronic pruritus from sulfur mustard	Traditional (23–28 yrs post exposure)	Investigator-blinded, randomized, controlled	Both decreased pruritus, visual analog score, and Dermatology Quality of life index. No placebo group	(17)
ANIMAL STUDIES					
Iodine	Oxidation of substance P by iodine induces protective factor nonapeptide	After heat-induced skin damage	Guinea pigs	Induces expression of protective nonapeptide that reduces inflammation in when transferred to another host	(35)
Silymarin	Supposedly reduces inflammatory cytokine production	Pretreatment and traditional	BALB-C mice	Reduced DNCB, croton oil, and TPA-induced ear swelling, measured by changes in ear thickness, both prophylactically and post irritation	(35)
Laminaria ocroleuca	Anti-inflammatory	Pre-treatment	BALB-C mice	Inhibits DNCB-induced ear swelling	(36)
Alchornea cordifolia	Reduced croton oil-induced edema in mice	Traditional therapy	Controlled randomized trial	African plant used traditionally for treatment of bacterial, fungal, parasitic, and inflammatory disorders	(37)
Steroid/NSAID combination: Adexona/Voltaren	Significantly reduced edema, erythema, and inflammatory markers (PGE) due to sulfur mustard in mice	Traditional therapy	Open, controlled	Moderately more effective against second phase of skin injury (extensive epithelial damage with vesiculation and necrosis) than above treatment	(14)
"Triple therapy" with indomethacin, promethazine, and niacinamide (NSAID, antihistamine, vasoconstrictor)	Significantly reduced sulfur mustard-induced erythema in hairless guinea pigs	Pre-treatment	Open, controlled	Skin injury still occurred; suggested therapy only effective against initial phase of skin irritation	(15)
Methysergide	Serotonin antagonist	Traditional therapy	Open, randomized, controlled	Slight reduction in AA-induced ear edema	(38)
Lipoxygenase inhibitors (zileutin, MK886)	Inhibition of arachidonic acid	Traditional therapy	Open, randomized, controlled	Potently suppressed arachidonic acid-induced ear edema in mice	(38)
Indomethacin, ketoprofen	Inhibition of cyclooxygenase	Traditional therapy	Open, randomized, controlled	Anti-edema effects reduced by topical application of PG-E2	(38)
Capsular polysaccharides of cyanobacteria	Anti-inflammatory agents: inhibited the croton oil-induced edema in male albino mice	Traditional therapy	Controlled randomized trial	Required 6 h application: not all strains effective: dose-dependent effects	(39)

Abbreviations: BID, Twice a day; ESCD, European Society of Contact Dermatitis; PG-E, Prostaglandin E; ICD, irritant contact dermatitis; NSAIDs, nonsteridal anti-inflammatory drug; NON, nonanoic acid; SLS, sodium lauryl sulfate; TEWL, trans-epidermal water loss.

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61 Validation and regulatory acceptance of dermatotoxicology methods: Recent progress and the role of NICEATM and ICCVAM

William S. Stokes and Judy Strickland

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is an interagency committee consisting of 15 U.S. Federal regulatory and research agencies that use, generate, require, or disseminate safety-testing information (Table 61.1). The committee was established in 1997 to coordinate the interagency evaluation of the scientific validity of new, revised, and alternative methods proposed for regulatory safety testing. The ICCVAM Authorization Act of 2000 (1) established ICCVAM as a permanent interagency committee of the National Institute of Environmental Health Sciences under the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) with specific purposes and duties (Table 61.2). ICCVAM and NICEATM work collaboratively to promote the validation and regulatory acceptance of new, revised, and alternative test methods that are based on sound science and that will provide continued or improved protection of people, animals, and the environment while reducing, refining, and replacing the use of animals where scientifically feasible. NICEATM administers ICCVAM and provides scientific and technical support for ICCVAM activities. NICEATM organizes test method peer reviews and workshops in conjunction with ICCVAM and carries out independent validation studies on high priority test methods (2).

NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of U.S. Federal regulatory agencies (3). After comprehensive scientific evaluations with multiple opportunities for public comment, ICCVAM and NICEATM forward formal recommendations to Federal agencies on test method usefulness and limitations for regulatory testing (4,5) (Fig. 61.1).

VALIDATION AND REGULATORY ACCEPTANCE

In the United States, Federal laws require that new safety assessment methods proposed for regulatory decisions must be determined to be sufficiently valid and acceptable for their intended use (1). ICCVAM developed criteria for validation and regulatory acceptance that have been internationally harmonized (6,7) (Table 61.3). The criteria are general principles that should be appropriately addressed when considering the validity of new, revised, or alternative test methods. Flexibility is essential in interpreting and applying the criteria; the extent that each criterion should be addressed will depend on the intended use and nature of the test method (6,7). The criteria should be considered early in the test method development process in consultation with ICCVAM.

When new test methods are developed, they typically progress through three levels of standardization and validation (9,10). The first level is *technical validation*, which determines the extent that the technology used in the test method is capable of providing consistent and reproducible results when substances that generate data across the range of test system responses are tested repeatedly over time. The second level involves *biologic validation*, which determines the extent that the underlying biological responses assessed in the test system are correctly measured. For example, are the measured qualitative and quantitative responses indicative of the biologic response, or are there other factors that may be causing unrelated positive, negative, or altered responses? Once technical and biologic validity are established, and the test system is proposed for possible regulatory hazard or safety decision-making, the test system would progress to *regulatory validation*.

Regulatory validation involves assessing the accuracy and reliability of the test method for a specific purpose (3,6,7). Regulatory validation determines the usefulness and limitations of a test method for making specific regulatory safety or hazard decisions. Assessments of relevance typically characterize accuracy, sensitivity, specificity, and false positive and negative rates compared to results generated with one or more existing reference test methods. Reliability assessments determine whether reproducible results can be obtained in different laboratories when following a standardized and optimized test method protocol. Regulatory acceptance consideration involves reviewing the validation results to determine whether the intended use of the method will provide equivalent or improved protection compared to existing methods (1). Since their establishment, NICEATM and ICCVAM have contributed to the evaluation of 51 test methods that have been accepted or endorsed by national and international authorities (http://iccvam.niehs.nih.gov/about/accept.htm).

THE MURINE LOCAL LYMPH NODE ASSAY (LLNA)

The first dermatotoxicology test method evaluated and recommended by ICCVAM was the murine local lymph node assay (LLNA) (11–14). The LLNA is a mechanism-based assay for

TABLE 61.1
ICCVAM Member Agencies

Regulatory Agencies	Research Agencies
Consumer Product Safety Commission	Agency for Toxic Substances and Disease Registry
Department of Agriculture[a]	Department of Defense
Department of the Interior[a]	Department of Energy
Department of Transportation	National Cancer Institute
Environmental Protection Agency[a]	National Institute for Occupational Safety and Health-CDC
Food and Drug Administration[a]	National Institute of Environmental Health Sciences
Occupational Safety and Health Administration	National Library of Medicine
	National Institutes of Health, Office of the Director

[a]Also has a research component.
Abbreviations: ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods.

TABLE 61.2
The purposes and duties of ICCVAM from the ICCVAM Authorization Act of 2000.

Purposes of ICCVAM
Increase the efficiency and effectiveness of U.S. Federal agency test method review
Eliminate unnecessary duplication of effort and share experience among U.S. Federal regulatory agencies
Optimize use of scientific expertise outside the U.S. Federal Government
Ensure that new and revised test methods are validated to meet the needs of U.S. Federal agencies
Replace, reduce, or refine (decrease or eliminate pain and distress) the use of animals in testing where feasible
Duties of ICCVAM
Review and evaluate proposed new, revised, and alternative test methods
Facilitate interagency and international harmonization of test methods
Facilitate and provide guidance on test method development, validation criteria, and validation processes
Promote the acceptance of scientifically valid test methods
Submit test recommendations to U.S. Federal agencies
Consider requests from the public for review and evaluation of test methods for which there is evidence of scientific validity

Abbreviations: ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; U.S., United States.

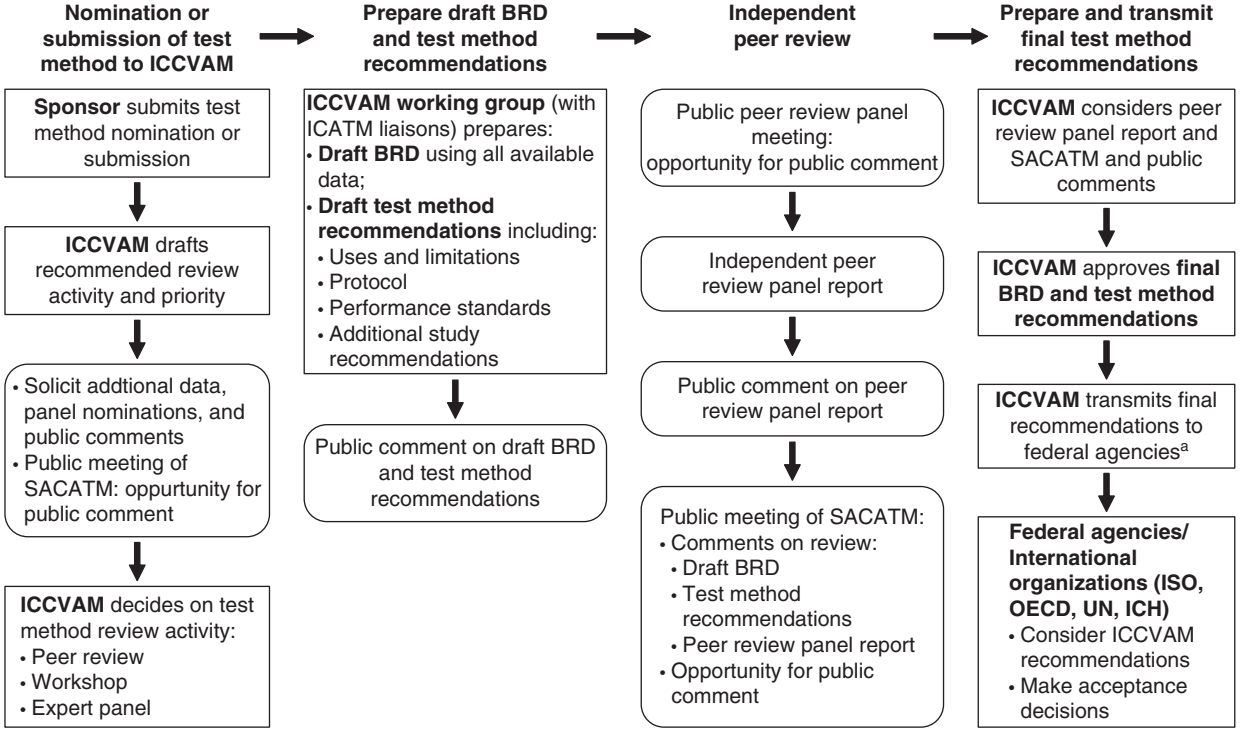

FIGURE 61.1 ICCVAM Test Method Evaluation Review Process. [a]Transmittal through the Secretary, Department of Health and Human Services, or designee. *Abbreviations*: BRD, background review document; ICATM, International Cooperation on Alternative Test Methods (members include ICCVAM, European Centre for the Validation of Alternative Methods, Environmental Health Science and Research Bureau within Health Canada, Japanese Center for the Validation of Alternative Methods, and Korean Center for the Validation of Alternative Methods); ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; ISO, International Organization for Standardization; OECD, Organisation for Economic Co-operation and Development; SACATM, Scientific Advisory Committee on Alternative Toxicological Methods; UN, United Nations.

TABLE 61.3
Summary of ICCVAM Criteria for Validation and Regulatory Acceptance of New, Revised, or Alternative Test Methods[a]

Validation Criteria	Regulatory Acceptance Criteria
Clear statement of proposed use	Fits into the regulatory testing structure
Biological basis/relationship to effect of interest	Adequately predicts the toxic endpoint of interest
Formal detailed protocol	Generates data useful for risk assessment
Reliability assessed	Adequate data available for specified uses
Relevance assessed	Robust and transferable
Limitations described	Time and cost-effective
All data available for review	Adequate animal welfare consideration
Data quality: ideally GLPs	
Independent scientific peer review	

[a]This table provides a summary of the ICCVAM validation and regulatory acceptance criteria. For a complete description of the criteria, please refer to the report: Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods. NIH Publication. No. 97–3981. Research Triangle Park, NC: National Institute of Envrionmental Health Science; 1997 (8).
Abbreviations: GLPs, good laboratory practices.

allergic contact dermatitis (ACD) testing that uses fewer animals and eliminates pain and distress compared to traditional guinea pig (GP) ACD assays such as the Buehler Test and the Guinea Pig Maximization Test (14). The LLNA measures changes to one of the key biological pathway events that is required for the development of chemically induced ACD: lymphocyte proliferation in the lymph nodes that drain the skin area where the test article is repeatedly applied. The LLNA only assesses events that occur during the induction phase of ACD, and therefore avoids the need for a later chemical challenge exposure necessary to elicit an allergic response. The endpoint for the traditional GP assays is elicitation, which is characterized by erythema and edema that occurs 24 to 72 hours after a challenge exposure to a sensitizing substance (15).

The LLNA procedure involves applying test or control substances (25 μl) to the dorsum of each mouse ear on days 1, 2, and 3 (16,17) (Fig. 61.2). On day 6, radiolabeled methyl thymidine or iododeoxyuridine is administered via intravenous injection. The mice are humanely killed 5 hours later. The auricular lymph nodes are excised, and a single cell suspension is prepared for scintillation counting. Actively dividing lymphocytes incorporate the radiolabeled markers, which are measured quantitatively and expressed as disintegrations per minute (DPM). The mean of the results from each dose group of treated mice are compared to the mean value for the control group of mice, which is expressed as a ratio referred to as the stimulation index (SI). Chemicals that generate an SI of 3.0 or higher are considered positive for ACD hazard potential and those with an SI less than 3.0 are considered negative.

The comprehensive ICCVAM evaluation of the validation status and subsequent recommendations for regulatory applications of the LLNA served a key role in the rapid international acceptance and widespread use of the LLNA (11–14). The initial LLNA test method data submission to ICCVAM was made by Drs Frank Gerberick (Procter and Gamble, USA), David Basketter (Unilever, UK), and Ian Kimber (Zeneca, UK) (14). The LLNA was already accepted as a screening test for which a positive response was accepted, but a negative response required confirmation in a GP test. The sponsors asked ICCVAM to evaluate the validity of the LLNA as a stand-alone substitute to the GP methods traditionally used to classify substances that may cause ACD. The sponsors proposed that both positive and negative responses in the LLNA should be accepted without any confirmatory test.

The sponsors submitted LLNA data for 209 chemicals representing a wide range of chemical and product classes; 62 substances had LLNA, GP, and human data (14). The accuracy of the LLNA (72% [45/62]) was the same as the accuracy of the traditional GP tests for predicting ACD in humans (11,13). As a result of the evaluation, ICCVAM recommended the LLNA to U.S. Federal agencies as a valid substitute for the accepted GP test methods for assessing the ACD hazard potential of many types of substances (11,13,14). At that time, the LLNA was not recommended for testing: metals due to some false negative results; mixtures, due to the lack of data; and aqueous preparations due to their lack of appropriate contact time with the skin. ICCVAM also concluded that the LLNA provided several advantages over GP test methods, including animal refinement by avoiding the pain and distress that could occur during the elicitation phase of sensitization, reduction in the use of animals, increased efficiency in terms of less time to perform, and the provision of dose-response information (12,14). Based on the ICCVAM evaluation, the LLNA was accepted nationally and internationally and incorporated into the following test guidelines for the assessment of ACD hazard potential:

- U.S. Environmental Protection Agency Health Effect Testing Guidelines on Skin Sensitization (16)
- Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 429. Skin Sensitisation: Local Lymph Node Assay (18)
- International Organization for Standardization 10993-10: Tests for Irritation and Delayed-type Hypersensitivity (19)

The LLNA is now commonly used worldwide and is the preferred method to determine the ACD hazard for most types of substances (20,21). This original LLNA test method protocol is now often referred to as the traditional LLNA, as new versions of the LLNA that use fewer animals and nonradioactive markers of lymphocyte proliferation were subsequently developed, validated, and accepted by regulatory authorities. These will be discussed in greater detail in this chapter.

LLNA Test Method Performance Standards

Shortly after the LLNA was approved as a regulatory test method for ACD, efforts were initiated to modify the LLNA to provide improved accuracy and efficiency and to develop versions that did not require the use of radioisotopes. ICCVAM recognized the need to develop criteria that could be used to more efficiently evaluate the validity of modified versions of adequately validated and accepted test methods such as the LLNA. NICEATM and ICCVAM subsequently developed and published the concept of performance standards, which are standardized criteria that can be used to more efficiently evaluate the validity of a test method that is functionally and mechanistically analogous to a validated method, such as the LLNA (3,22–24).

Following a request by the U.S. Consumer Product Safety Commission (CPSC) to evaluate the validity of several modified versions of the LLNA in 2007, ICCVAM determined that performance

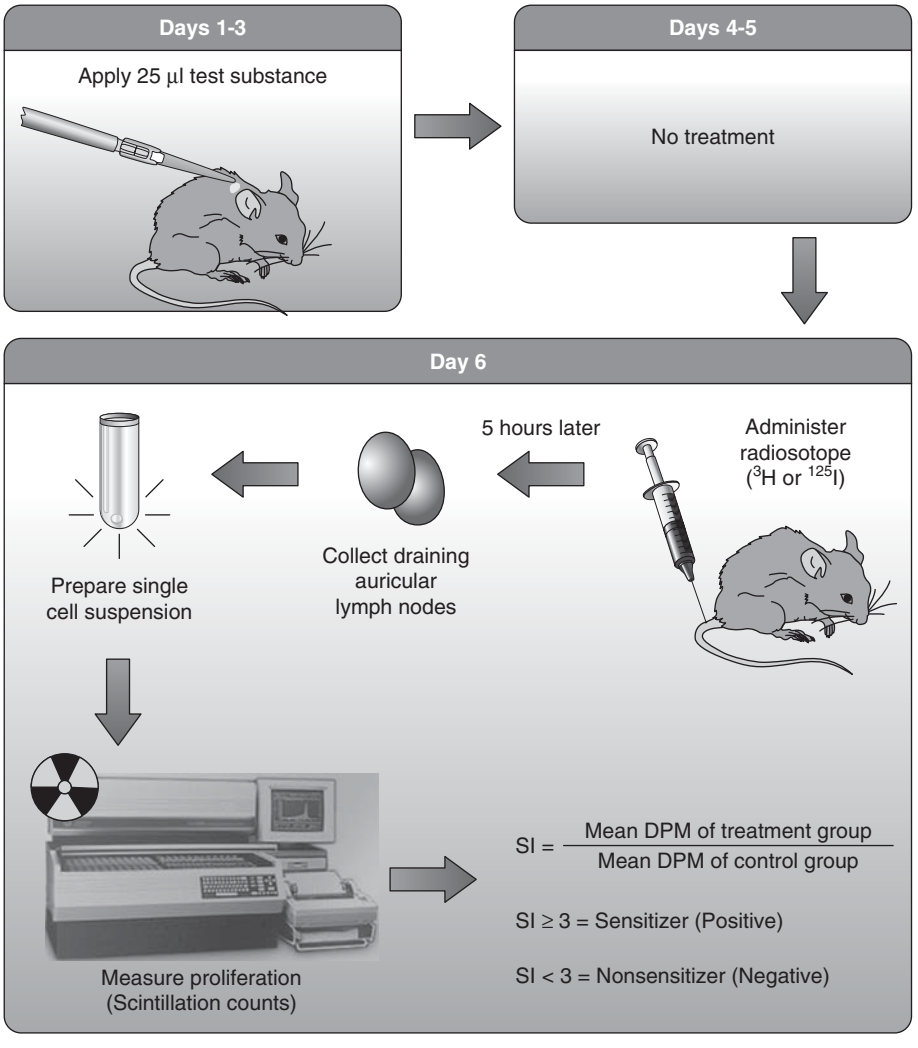

FIGURE 61.2 Test Method Protocol for the Traditional LLNA. Abbreviations: DPM, disintegrations per minute; LLNA, murine local lymph node assay; SI, stimulation index.

standards should be developed for the LLNA that could be used to evaluate these methods and any other improved versions that might be developed. ICCVAM subsequently worked together with the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM) to develop internationally harmonized LLNA performance standards that could be proposed for inclusion in a revision of OECD TG 429, which describes the use of the LLNA for determining ACD hazard potential of chemicals and other products (25).

Performance standards consist of: 1) essential test method components, 2) reference substances, and 3) standards for accuracy and reliability that the proposed test method should meet or exceed (24).

Essential Test Method Components for Modified LLNA Methods

For a modified LLNA method to be eligible for evaluation using the performance standards, it must include the essential test method components listed in Table 61.4 (25). These essential test method components ensure that the same biological effect is being measured and indicates that the modified LLNA method is functionally and mechanistically comparable to the traditional LLNA.

TABLE 61.4
Essential Test Method Components for Validation of Modified LLNA Methods

Modified methods to be evaluated using the LLNA performance standards must include:
The test substance must be applied topically to both ears of the mouse.
Lymphocyte proliferation must be measured in the lymph nodes draining the site of test substance application.
Lymphocyte proliferation must be measured during the induction phase of skin sensitization.
The highest dose selected must be the maximum soluble concentration that does not induce systemic toxicity and/or excessive local irritation.
A vehicle control must be included in each study, and, where appropriate, a positive control should also be used.
A minimum of four animals per dose group must be included.
Either individual or pooled animal data may be collected.

Note: Collection of individual animal data is recommended by ICCVAM, and also required by several regulatory authorities.
Abbreviations: ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA, murine local lymph node assay.

TABLE 61.5
Reference Substances for Validation of Modified LLNA Methods

Substance Name	LLNA	Vehicle	EC3[a]	N[b]	Guinea Pig[c]	Human
CMI/MI	+	DMF	0.009	1	+	+
2,4-Dinitrochlorobenzene	+	AOO	0.049	15	+	+
4-Phenylenediamine	+	AOO	0.11	6	+	+
Cobalt chloride	+	DMSO	0.6	2	+	+
Isoeugenol	+	AOO	1.5	47	+	+
2-Mercaptobenzothiazole	+	DMF	1.7	1	+	+
Citral	+	AOO	9.2	6	+	+
Hexyl cinnamic aldehyde	+	AOO	9.7	21	+	+
Eugenol	+	AOO	10.1	11	+	+
Phenyl benzoate	+	AOO	13.6	3	+	+
Cinnamic alcohol	+	AOO	21	1	+	+
Imidazolidinyl urea	+	DMF	24	1	+	+
Methyl methacrylate	+	AOO	90	1	+	+
Chlorobenzene	-	AOO	NC	1	-	-[d]
Isopropanol	-	AOO	NC	1	-	-
Lactic acid	-	DMSO	NC	1	-	-[d]
Methyl salicylate	-	AOO	NC	9	-	-
Salicylic acid	-	AOO	NC	1	-	-
Optional Substances to Demonstrate Improved Performance Relative to the LLNA						
Sodium lauryl sulfate	+	DMF	8.1	5	-	-
Ethylene glycol dimethacrylate	+	MEK	28	1	-	+
Xylene	+	AOO	95.8	1	NA	-
Nickel chloride	-	DMSO	NC	2	+	+

[a]Arithmetic means where the number of LLNA studies > 1.

[b]Number of LLNA studies from which data were obtained.

[c]Results obtained from Guinea Pig Maximization Test and/or Buehler Test.

[d]Presumed to be a nonsensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of human sensitization were located.

Abbreviations: AOO, acetone: olive oil (4:1);CMI/MI, 3:1 5-chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one ("KathonCG"); DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EC3, estimated concentration needed to produce a stimulation index of 3; LLNA, murine local lymph node assay; MEK, methyl ethyl ketone; NA, not available; NC, not calculated because the stimulation index < 3.

Reference Substances for Evaluating Modified LLNA Methods

The validation of modified LLNA methods must include a minimum of 18 reference substances (25). The criteria used to select chemicals as reference substances were: (*i*) commercial availability; (*ii*) existing LLNA, and GP data and human data where possible; and (*iii*) representative of the types of substances typically tested for skin sensitization potential and the range of negative and positive responses observed for the LLNA (Table 61.5). Four additional substances, which tested false negative or false positive in the LLNA, were provided as optional substances that could be tested to demonstrate equivalent or superior performance to the traditional LLNA.

Accuracy and Reliability Standards for Modified LLNA Methods

The LLNA performance standards define the accuracy that a modified LLNA test method should meet to be considered as equivalent to the LLNA. Accuracy is assessed by testing the 18 minimum recommended positive and negative reference substances (25). The proposed modified LLNA method should result in the same classification as the traditional LLNA result. If the modified LLNA does not correctly classify all of the reference substances, a rationale for the misclassification and appropriate additional data (e.g., test results that provide correct classifications for substances with similar properties to those of the misclassified substance) could be considered to demonstrate equivalent performance. Limitations to the applicability domain could also be imposed for categories of chemicals for which there is poor predictivity.

Performance standards also define the reliability that should be achieved by a proposed new modification of the LLNA. Reliability is the degree to which a test method can produce results consistently over time within a single laboratory, referred to as intralaboratory reproducibility, and among different laboratories, referred to as interlaboratory reproducibility (24,25). To determine intralaboratory reproducibility, the LLNA performance standards recommend testing hexyl cinnamic aldehyde (HCA) in the proposed test method on four separate occasions with at least one week between tests. Acceptable intralaboratory reproducibility is achieved with ECt values (estimated concentration needed to produce a stimulation index at a specific threshold value) between 5% and 20%, which represents the range of 0.5x to 2.0x the mean EC3 for HCA (9.7%) in the traditional

LLNA (25). To determine interlaboratory reproducibility, ICCVAM recommends testing HCA and 2,4-dinitrochlorobenzene (DNCB) once each in 3 different laboratories. Acceptable interlaboratory reproducibility is demonstrated with ECt values of 5% to 20% for HCA and 0.025% to 0.1% for DNCB. It is important to note that the number of chemicals included in the LLNA performance standards to assess accuracy and reliability are typically smaller than performance standards developed for in vitro methods.

EXPANDED APPLICATIONS, NEW VERSIONS, AND IMPROVEMENTS TO THE LLNA

The widespread use of the LLNA following its evaluation by ICCVAM in 1998 led to a number of improvements, new versions, and expanded applications of the LLNA. New versions were developed to reduce the use of animals by reducing the number of doses tested and to measure cell proliferation using techniques that do not require radiolabeled reagents (26–33). There were also new data available suggesting that the applicability domain of the LLNA could be expanded to include mixtures, aqueous substances, and metals (34–36). In addition, there were suggestions that the LLNA could be used to categorize the potency of skin-sensitizing substances (26). Thus, in 2007, the U.S. CPSC requested that ICCVAM evaluate several expanded applications, new versions, and improvements to the LLNA. The nominated activities included the following:

- The use of the LLNA to test mixtures, aqueous solutions, and metals
- The reduced LLNA (rLLNA)
- Nonradioactive LLNA test method protocols
- The LLNA as a stand-alone assay for classification of ACD potency

ICCVAM and NICEATM subsequently conducted comprehensive evaluations of the validation status of these test methods and applications, and prepared test method evaluation reports and recommendations that were forwarded to U.S. agencies and international organizations for consideration. NICEATM and ICCVAM coordinated its evaluations in collaboration with ECVAM, JaCVAM, and Health Canada in accordance with an international cooperation agreement on alternative test methods (37).

Expanded Applicability Domain of the LLNA

In the original ICCVAM evaluation of the LLNA in 1999, ICCVAM recommended that certain substances not be tested for ACD hazard using the LLNA due to lack of data, poor LLNA predictivity regarding their potential to cause ACD, or technical issues. The LLNA was not recommended for testing: 1) metals, due to some false negative results; 2) mixtures, for which there were no data; and 3) aqueous substances, which do not adhere to the skin. After a number of years of international experience with the LLNA and the accumulation of additional data, the usefulness of the LLNA for testing these types of substances was reevaluated in 2008 in response to the request from the U.S. CPSC (38).

NICEATM divided substances that could be considered as mixtures by product groups: pesticide formulations, dyes, and natural complex substances (i.e., fragrance oil and extracts) (38). After examining the performance of the LLNA against the available GP and/or human data, ICCVAM recommended that the LLNA could be used for testing: metal compounds, with the exception of nickel; pesticide formulations; dyes; natural complex substances; and substances tested in aqueous solutions. ICCVAM recommended that any substance, other than those specifically excluded, could be tested in the LLNA, unless the substance has unique physicochemical properties that may interfere with the ability of the LLNA to identify it as a sensitizing substance (38). Inconsistent results for nickel compounds obtained with the traditional LLNA suggest that the LLNA may not be suitable for testing substances containing nickel. U.S. Federal agencies concurred with the ICCVAM recommendations (http://iccvam.niehs.nih.gov/methods/immunotox/llna.htm). The U.S. Environmental Protection Agency (EPA) updated their policy to also accept LLNA data for testing pesticide products, in addition to their previous acceptance of results for technical grade active ingredients (39).

The Reduced LLNA Test Method Protocol

The reduced LLNA (rLLNA) is a modification of the LLNA that reduces animal use by 40% per test (33,40,41). The only difference between the test method protocols for the multidose LLNA and the rLLNA is the number of dose levels tested. In the multidose LLNA, at least three dose levels are tested, with the highest dose based on the maximum soluble concentration that avoids excessive local irritation and/or systemic toxicity. Only the highest dose of a substance is tested in the rLLNA (33,40). At the request of the U.S. CPSC, ICCVAM evaluated the usefulness and limitations of the rLLNA in 2009.

The evaluation involved a retrospective comparison of rLLNA results to multidose LLNA results (40). The rLLNA results were those generated from the highest dose of the multidose LLNA tests. The evaluation database included 457 unique substances tested in 471 multidose LLNA studies. Compared to the multidose LLNA, the rLLNA had an accuracy of 98.7% (465/471), a false positive rate of 0% (0/153), and a false negative rate of 1.9% (6/318). For the 6 substances that tested false negative in the rLLNA, the multidose LLNA classifications of the substances as skin sensitizers were based on low- or mid-doses that produced SI ≥ 3, whereas the highest doses tested produced SI <3. All 6 substances produced weak responses in the multidose LLNA; all SI values were <4. Because the rLLNA and traditional LLNA used identical protocols and the data sets used to evaluate their accuracy were similar, the intra- and interlaboratory reliability of the rLLNA was deemed to be similar to that of the multidose LLNA (40).

ICCVAM concluded that the scientific validity of the rLLNA had been adequately evaluated and that its performance was sufficient to distinguish between skin sensitizers and nonsensitizers if dose-response information was not required (40). ICCVAM recommended that the rLLNA should be used routinely to determine ACD potential of chemicals and products *before* conducting the traditional LLNA to minimize the number of animals used for ACD testing (Fig. 61.3). This is because the majority of products and chemicals are expected to produce negative results (42). Negative substances can therefore be classified as nonsensitizers and positive substances can be classified as sensitizers with 40% fewer animals. The exception to always using the rLLNA is for testing situations where dose-response information is required and there is evidence suggesting that the substance is likely to be a sensitizer. In such situations, these substances should initially be tested in the multidose LLNA.

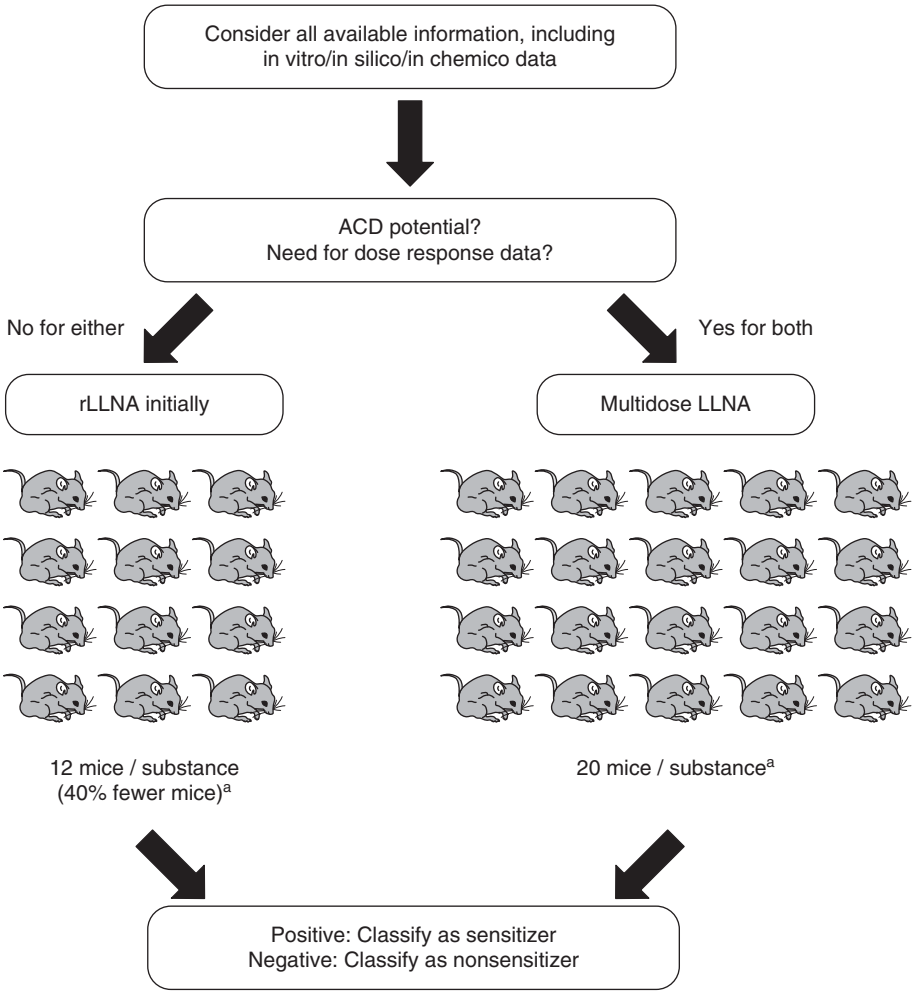

FIGURE 61.3 Decision Strategy for Using the rLLNA. [a]Four animals each in the following groups: negative control, positive control, and treatment group(s). *Abbreviations*: ACD, allergic contact dermatitis; rLLNA, reduced murine local lymph node assay LLNA.

Because of a small possibility of a false negative result in the rLLNA, negative results should prompt a weight-of-evidence evaluation of all available information (40). Items that could be considered in such an evaluation include factors that could reduce skin absorption at the high dose, structural relationship to known sensitizers, test results with similar substances, peptide-binding activity, molecular weight, and other in vitro/in silico/in chemico data.

U.S. Federal agencies concurred with the ICCVAM recommendations for using the rLLNA (http://iccvam.niehs.nih.gov/methods/immunotox/rLLNA.htm#agencyresponses). The U.S. EPA recently published a policy to accept rLLNA data in situations where dose-response data are not needed, such as when results are expected to be negative (39).

Recent Updates to the LLNA Test Method Protocol

Following the 2008 review of the LLNA test method protocol, ICCVAM updated it to include several improvements and approaches that could further reduce animal use (25). The updated LLNA protocol includes the following key revisions: 1) reduction in the minimum number of animals per dose group to 4 rather than 5; 2) improved guidance on the importance of collecting data from individual animals rather than pooling lymph nodes from all animals in a dose group; 3) guidance on when it may be appropriate to reduce the number of positive control animals, including statistical analysis to justify the reduction; and 4) detailed guidance on conducting a dose range finding study and criteria for selection of the highest dose based on local irritation and systemic toxicity (25). The reduction in the number of animals per group was based on a comparison of LLNA results using groups of five or more animals to LLNA results for groups of four animals (25,43). This change will allow for individual animal data to be collected in all testing situations, since previously five animals were required per group for this approach, whereas only four animals were required for pooled data. Pooled data is discouraged because a review of actual data revealed the possibility of low outlier values that could result in false negative results (40). Outlier analysis is also now recommended whenever unusually high or low values are observed. The detailed guidance on selecting the highest dose to test insures that the maximum dose is uniformly selected and appropriate. Regulatory agencies have confidence in negative results only when supported by sufficient rationale for the highest dose tested, and are less likely to require additional testing if the basis for the highest dose is clear and supported by relevant data.

A revised OECD TG for the LLNA was adopted in June 2010 (17). The updated TG incorporates the key additions to the updated ICCVAM LLNA protocol (25), the ICCVAM recommended rLLNA (40), the ICCVAM recommendations for an expanded LLNA applicability domain (38), and the ICCVAM recommended LLNA performance standards (25).

Two New Non-radioisotopic LLNA Test Methods

Because the LLNA uses radioisotopes to quantify cell proliferation induced by potential sensitizers, its use is limited to laboratories qualified to use radioactive reagents. The recent adoption and availability of two non-radioisotopic LLNA test methods now enables broader use of the LLNA (44,45). Laboratories that are not approved to use radioisotopes can now use one of the newly approved non-radioisotopic LLNA test methods instead of the traditional GP test methods. These new LLNA methods now allow for use of the LLNA for nearly all skin sensitization–testing situations, resulting in significantly expanded animal welfare benefits of the LLNA in terms of reduced animal use and avoidance of pain and distress compared to the GP test methods. The nonradioactive LLNA test methods also offer environmental advantages by avoiding the generation of radioactive wastes. NICEATM and ICCVAM evaluated two non-radiolabeled LLNA methods (44,45).

The LLNA: DA Test Method

The LLNA: DA ("D" for Daicel Chemical Industries, Ltd., and "A" for ATP) is a nonradioactive LLNA method developed by Idehara and colleagues at Daicel Chemical Industries, Ltd. (28,29). The LLNA: DA measures increases in ATP content in the draining auricular lymph nodes removed from treated and control mice. ATP content is quantified using a luciferin–luciferase assay to measure bioluminescence. Because ATP content correlates with living cell number, its measurement serves as an indicator of cell number at the time of sampling (46). The protocol for the LLNA: DA is similar to that for the LLNA (Fig. 61.4). A minimum of four mice are used per dose group, with at least three dose groups, plus concurrent vehicle and positive control groups (44).

Test and control substances (25 µl) are applied to the dorsum of each ear on days 1, 2, 3, and 7, one hour after pretreatment with 1% sodium lauryl sulfate (SLS) (44). The SLS increases the absorption of the test substance across the skin (47). On day 8, the mice are humanely killed, the lymph nodes are excised, and a lymph node cell suspension is prepared for each mouse (44). ATP content, which is proportional to the light produced by the luciferin–luciferase reaction, is measured using a commercially available kit. An SI value for each treatment group is calculated as the ratio of the mean relative luminescence units for the treatment group compared to the mean relative luminescence units for the control group.

The 44 substances in the validation database represented a variety of chemical classes and product uses: 32 substances were LLNA skin sensitizers and 12 were LLNA nonsensitizers (44). NICEATM and ICCVAM evaluated multiple SI decision criteria

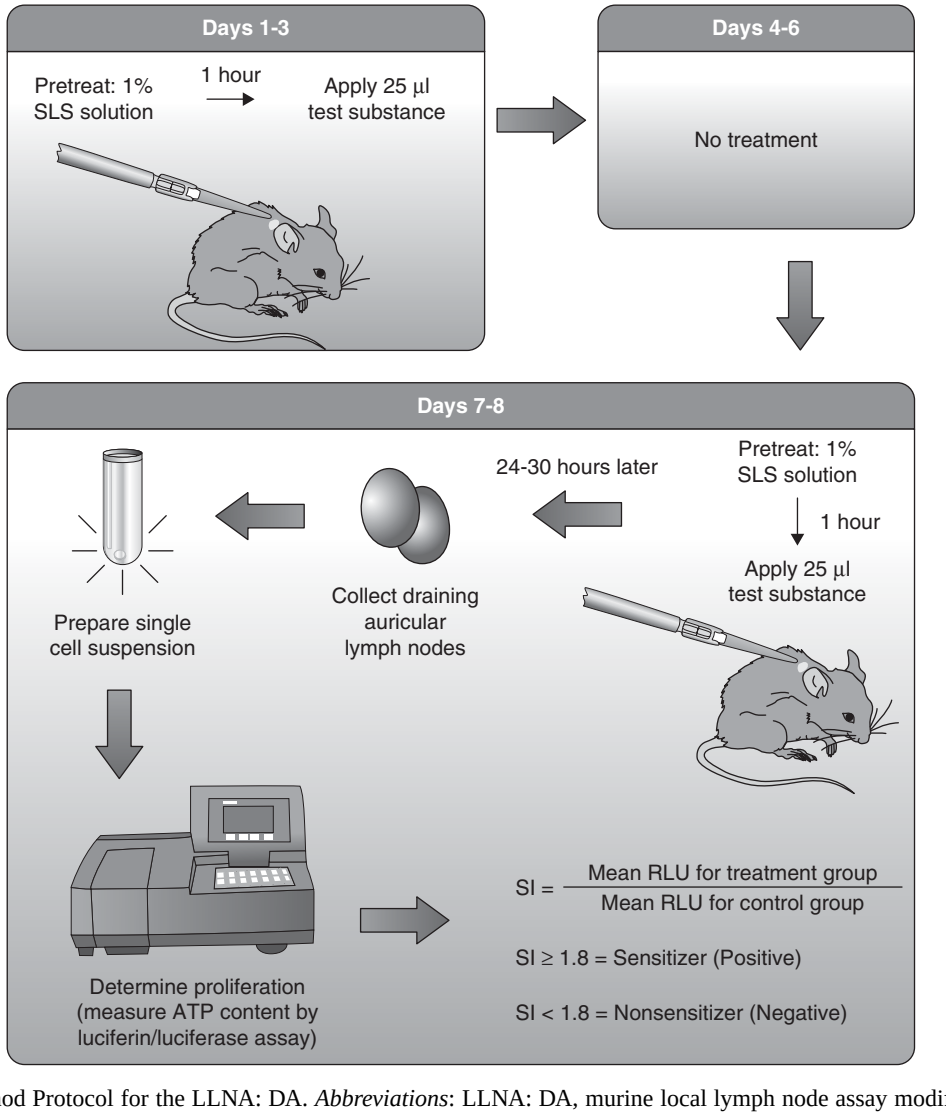

FIGURE 61.4 Test Method Protocol for the LLNA: DA. *Abbreviations*: LLNA: DA, murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; RLU, relative luminescence units; SI, stimulation index; SLS, sodium lauryl sulfate.

to identify the criterion that maximized accuracy with no false negative results, in comparison to results of the LLNA (Fig. 61.5). Optimal LLNA: DA performance was achieved using SI ≥1.8 to classify sensitizers. Compared to the traditional LLNA, accuracy was 93% (41/44), with a false positive rate of 25% (3/12) and a false negative rate of 0% (0/32). The reproducibility assessment of the LLNA: DA results for 14 substances (10 LLNA sensitizers and 4 LLNA nonsensitizers) showed that the results were concordant for 78% (11/14) of the substances tested in 3 to 18 tests across up to 18 different laboratories.

The reduced protocol for the LLNA: DA, the rLLNA: DA, requires only a single high dose group similar to the radioactive rLLNA and reduces the use of animals by 40% (44). The concentration tested should be the maximum concentration that does not induce overt systemic toxicity and/or excessive local skin irritation (44). Accuracy of the rLLNA: DA compared to the multidose LLNA: DA was 98% (121/123), with a false positive rate of 0% (0/33) and a false negative rate of 2% (2/90). In testing situations that do not require dose-response information, the rLLNA: DA should be considered for use as a reduced test method protocol.

ICCVAM recommended the LLNA: DA for identifying potential skin sensitizers and nonsensitizers (44). Since there were no false negatives relative to the LLNA, ICCVAM recommended that SI ≥1.8 be used as the decision criterion to classify potential sensitizers. The applicability domain for the LLNA: DA is considered to be the same as the LLNA, unless there are properties associated with a class of materials that may interfere with the test method's accuracy. For example, the use of the LLNA: DA might not be appropriate for testing substances that affect the ATP content of cells (e.g., substances that function as ATP inhibitors). U.S. Federal agencies concurred with the ICCVAM recommendations (http://iccvam.niehs.nih.gov/methods/immunotox/llna.htm).

The LLNA: BrdU-ELISA Test Method

The LLNA: BrdU-ELISA ("BrdU" for bromodeoxyuridine and "ELISA" for enzyme-linked immunosorbent assay) is the second nonradioactive LLNA method evaluated and recommended by NICEATM and ICCVAM. The LLNA: BrdU-ELISA was developed by Takeyoshi and colleagues to assess lymphocyte proliferation in the draining auricular lymph nodes by measuring the incorporation of the thymidine analog, BrdU, into the DNA of dividing lymph node cells (27). The protocol of the LLNA: BrdU-ELISA is similar to that for the LLNA (Fig. 61.6). It uses a minimum of four mice per dose group, with at least three dose groups, plus concurrent vehicle and positive control groups (45). Test and control substances (25 μl) are applied to the dorsum of each ear on days 1, 2, and 3. On day 5, 5 mg BrdU in a volume of 0.5 mL physiological saline is administered via intraperitoneal injection. The mice are humanely killed on day 6. The auricular lymph nodes are excised, and a lymph node cell suspension is prepared individually for each mouse. A commercially available ELISA kit is used to assess BrdU incorporation, which is measured spectrophotometrically. An SI value is calculated for each treatment group as the ratio of the mean absorbance for the treatment group to the mean absorbance for the control group.

The validation database represented a variety of chemical classes and product uses (45). The 43 substances with existing LLNA data included 32 LLNA skin sensitizers and 11 LLNA nonsensitizers. NICEATM and ICCVAM evaluated multiple SI values to identify the decision criterion that maximized accuracy with no false negative results, in comparison to results of the traditional LLNA (Fig. 61.7). Optimal performance was achieved using SI ≥1.6 to classify sensitizers. Accuracy was 95% (41/43), with a

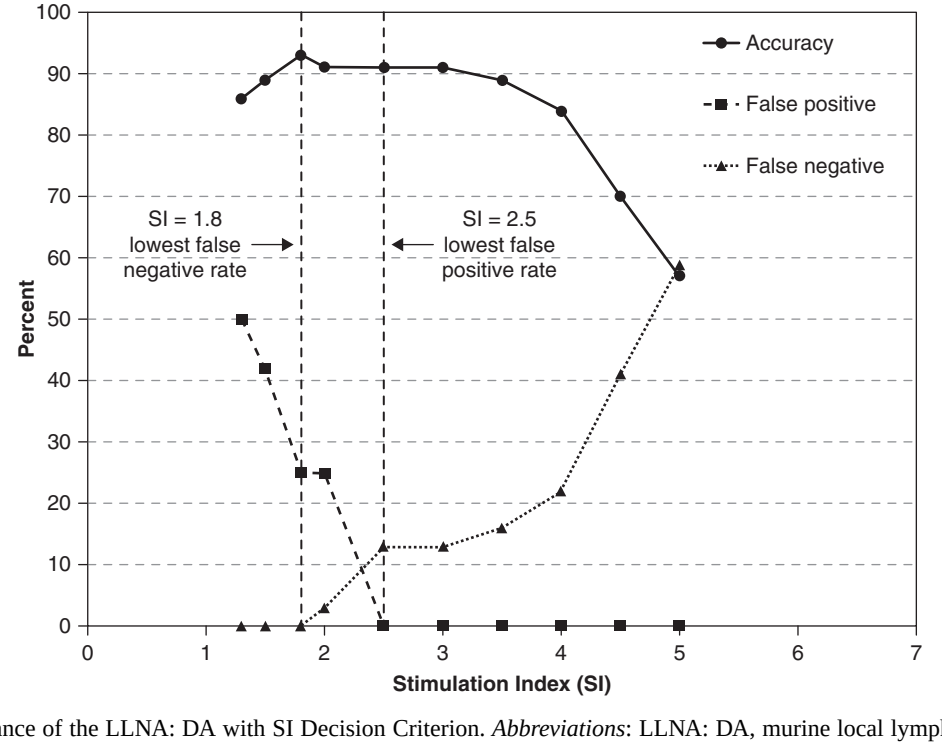

FIGURE 61.5 Performance of the LLNA: DA with SI Decision Criterion. *Abbreviations*: LLNA: DA, murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI, stimulation index.

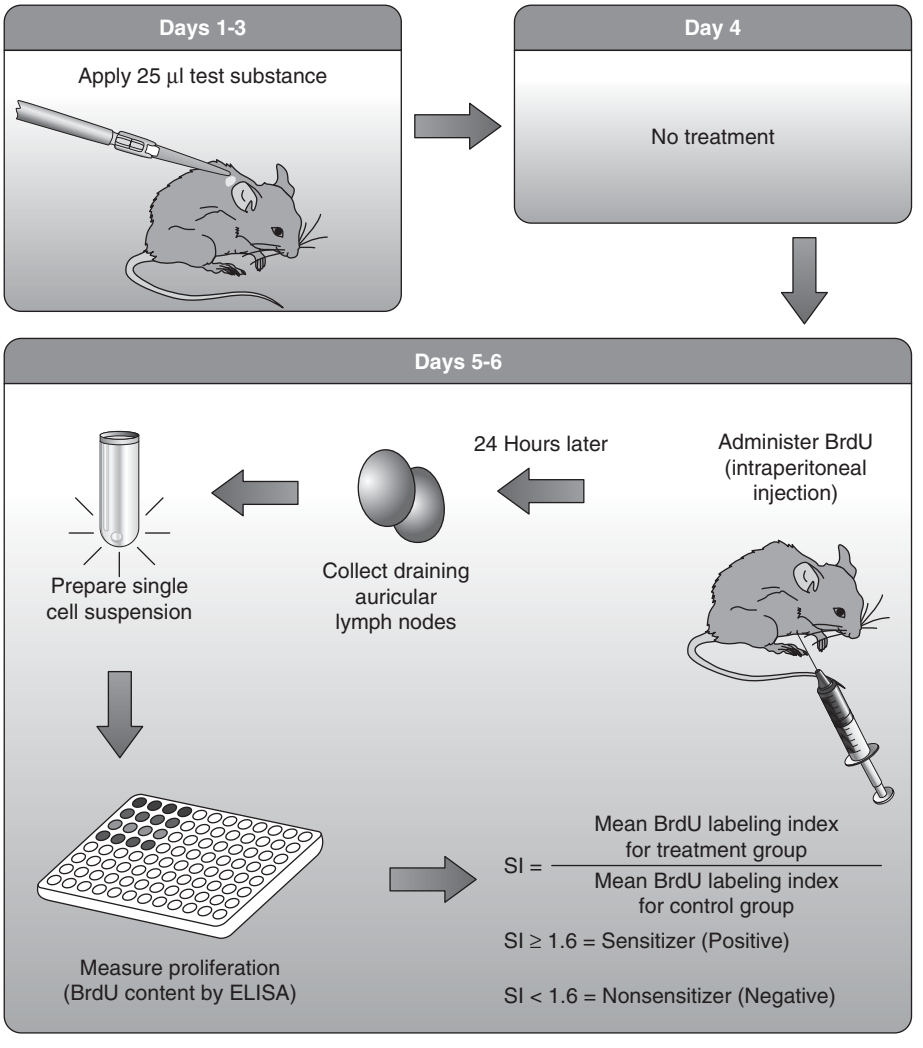

FIGURE 61.6 Test Method Protocol for the LLNA: BrdU-ELISA. *Abbreviations*: BrdU, bromodeoxyuridine; LLNA: BrdU-ELISA, murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI, stimulation index.

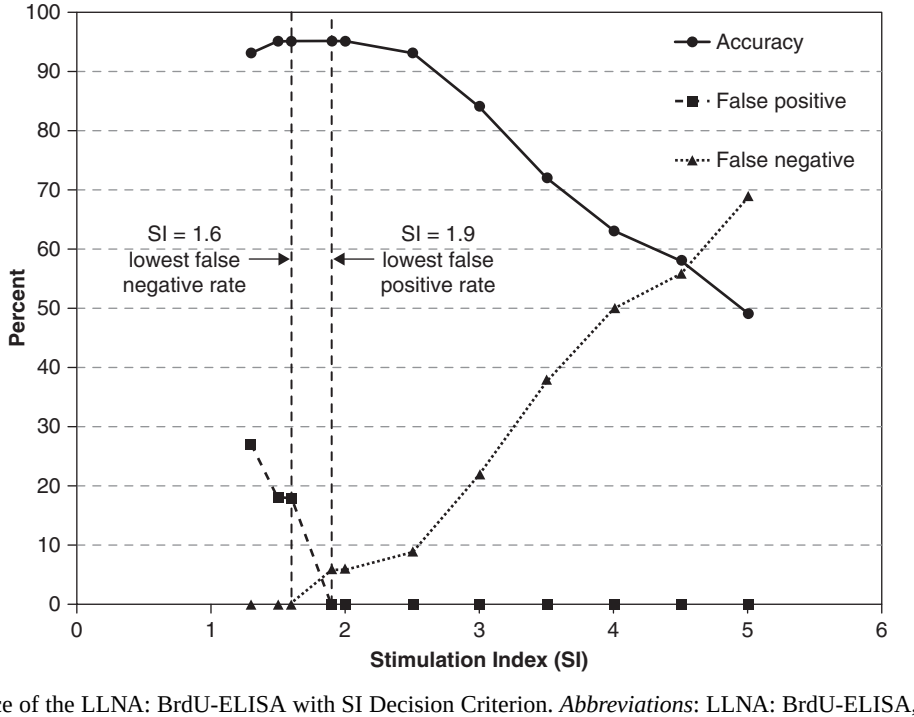

FIGURE 61.7 Performance of the LLNA: BrdU-ELISA with SI Decision Criterion. *Abbreviations*: LLNA: BrdU-ELISA, murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI, stimulation index.

false positive rate of 18% (2/11) and a false negative rate of 0% (0/32) when compared to the traditional LLNA. The reproducibility assessment of the LLNA: BrdU-ELISA was conducted using 18 substances (13 LLNA sensitizers and 5 LLNA nonsensitizers) with 2 to 12 test results across up to 8 different laboratories. The results were concordant for 78% (14/18) of the substances.

The LLNA: BrdU-ELISA also has a reduced protocol, the rLLNA: BrdU-ELISA, which requires only a single high dose group and that reduces the use of animals by 40% (45). The test substance concentration tested should be the maximum concentration that does not induce overt systemic toxicity and/or excessive local skin irritation (45). Accuracy of the rLLNA: BrdU-ELISA compared with the multidose rLLNA: BrdU-ELISA was 96% (82/85), with false positive and false negative rates of 0% (0/11) and 4% (3/74), respectively. In testing situations that do not require dose-response information, the rLLNA: BrdU-ELISA should be considered for use.

ICCVAM recommended the use of the LLNA: BrdU-ELISA for identifying potential skin sensitizers and nonsensitizers (45). Since there were no false negatives relative to the LLNA, ICCVAM recommended a SI ≥1.6 as the decision criterion for sensitizers. The applicability domain for the LLNA: BrdU-ELISA is considered to be the same as the LLNA, unless there are properties associated with a class of materials that may interfere with the test method's accuracy. The LLNA: BrdU-ELISA may also be useful in testing nickel compounds based on their correct identification as sensitizers in the validation study. U.S. Federal agencies generally concurred with the ICCVAM recommendations (http://iccvam.niehs.nih.gov/methods/immunotox/llna.htm).

Using the LLNA for ACD Potency Categorization

The U.S. CPSC, in accordance with requirements in the Federal Hazardous Substances Act (48), is the only regulatory agency in the United States that currently uses relative potency of sensitizers as a basis for hazard classification and labeling. The CPSC requires hazard labeling of only those products considered to be strong skin sensitizers (49). According to CPSC regulations, strong sensitizers are those substances that have a significant potential for causing hypersensitivity. Although the CPSC considers both human and animal data for the determination that a substance is a strong sensitizer, no quantitative criteria are currently applied. Rather, a weight-of-evidence assessment is made that considers frequency and severity of responses in exposed human populations, frequency and severity of animal responses, and the doses at which allergic reactions occur.

To establish the scientific validity of using quantitative LLNA data for potency categorizations, CPSC requested that ICCVAM evaluate the usefulness and limitations of the LLNA as a standalone test method for potency determinations. Using human ACD data and LLNA data for 136 substances, NICEATM determined the accuracy of the LLNA for classifying substances with known human skin sensitization potential into one of the three categories according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) criteria: GHS Subcategory 1A sensitizer, GHS Subcategory 1B sensitizer, or nonsensitizer (50).

A number of classification systems with quantitative criteria to categorize sensitizers based on relative potency have been proposed (26,51,52). However, only the GHS classification system has reached the level of international consensus for hazard classification and labeling (53,54). Under the GHS, when a relevant regulatory authority requires sub-categorization, the following subcategories are used for skin sensitizers:

- Subcategory 1A— strong skin sensitizers, for substances that frequently produce ACD in humans and/or have high potency in animals
- Subcategory 1B—"other" skin sensitizers, for substances that produce ACD with low to moderate frequency in humans and/or a low to moderate potency in animals.

The GHS provides quantitative human and animal test criteria for determining whether sensitizers should be classified into Subcategory 1A or 1B (Table 61.6) (54). Potency classification schemes using the LLNA are based on the effective concentration at the threshold response of SI = 3 (i.e., the EC3) (26,51,52,54).

The LLNA correctly identified 52% (14/27) of the human Subcategory 1A strong sensitizers using the GHS criterion of EC3 ≤2%, but 48% (13/27) were under-classified as Subcategory 1B, other than strong sensitizers (50). The GHS criterion for the LLNA correctly identified 71% (35/49) of the human Subcategory 1B other than strong sensitizers and over-classified 6% (3/49) as strong sensitizers. Increasing the EC3 cutoff increased the correct classification rate for Subcategory 1A sensitizers, but decreased the correct classification of Subcategory 1B sensitizers. The classification rate for nonsensitizers did not change with EC3 because the concordant LLNA nonsensitizers do not have EC3 values, and because the false positive LLNA results over-predict the human

TABLE 61.6
Human and LLNA Criteria for GHS Classification of Skin Sensitizers[a]

Category	LLNA	Human Response
1 (Sensitizer)	SI ≥ 3	Evidence that sensitization may be produced in a substantial number of persons
1A (Strong sensitizer)	EC3 ≤ 2%	Positive response at ≤ 500 μg/cm² (HRIPT or HMT induction threshold[b])
1B (Other sensitizer)	EC3 > 2%	Positive response at > 500 μg/cm² (HRIPT or HMT induction threshold[c])

[a]Although criteria for guinea pig data are also provided by the GHS, they were not used in this analysis.

[b]Human evidence for Subcategory 1A (strong) skin sensitizers can also include diagnostic patch test data where there is a relatively high and substantial incidence of reactions in a defined population in relation to relatively low exposure or other epidemiological evidence where there is a relatively high and substantial incidence of allergic contact dermatitis in relation to relatively low exposure.

[c]Human evidence for Subcategory 1B (other) skin sensitizers can also include diagnostic patch test data where there is a relatively low but substantial incidence of reactions in a defined population in relation to relatively high exposure or other epidemiological evidence, where there is a relatively low but substantial incidence of allergic contact dermatitis in relation to relatively high exposure.

Abbreviations: EC3, estimated concentration of a substance expected to produce a stimulation index of 3, which is the threshold value for a substance to be considered a sensitizer in the LLNA; GHS, Globally Harmonized System of Classification and Labeling of Chemicals (54); HMT, human maximization test; HRIPT, human repeat-insult patch test; LLNA, murine local lymph node assay; SI, stimulation index.

nonsensitizers at any EC3 value. Using LLNA EC3 ≤10% to classify substances as Subcategory 1A sensitizers correctly classified 89% (24/27) of the Subcategory 1A strong sensitizers.

Of the 27 Subcategory 1A human skin sensitizers in the validation database, 37% (10/27) produced EC3 values between 2% and 10% (50). Therefore, it is likely that a considerable number of Subcategory 1A human skin sensitizers within the broader population of chemicals may produce EC3 values within this range. These data suggest that substances with EC3 values between 2% and 10% should be considered as potential Subcategory 1A sensitizers unless there are data relevant to skin sensitization that indicate otherwise.

ICCVAM recommended that the LLNA could be used to categorize substances into GHS Subcategory 1A when the LLNA EC3 ≤ 2% because it correctly identified 52% (14/27) of the Subcategory 1A human skin sensitizers (50). However, because 48% (13/27) of the known Subcategory 1A human strong skin sensitizers have an EC3 > 2% or produce negative results in the LLNA, it should not be used alone to categorize a substance as GHS Subcategory 1B (other than strong sensitizer) when the substance produces an LLNA EC3 >2%. In such cases, additional information is required to categorize a substance as a GHS Subcategory 1B other than strong sensitizer.

The ICCVAM recommendations were transmitted to U.S. Federal agencies in June 2011. U.S. Federal agencies concurred with the ICCVAM recommendations (http://iccvam.niehs.nih.gov/methods/immunotox/LLNApotency.htm).

Future Directions

On April 27, 2009, Canada, the European Union, Japan, and the United States signed a Memorandum of Cooperation regarding the International Cooperation on Alternative Test Methods (ICATM) (37). The agreement provides for enhanced cooperation and collaboration between four national validation organizations: the NICEATM and ICCVAM, JaCVAM, ECVAM, and the Environmental Health Science and Research Bureau within Health Canada. On March 8, 2011, during the 50th Annual Meeting of the Society of Toxicology, the Korean Center for the Validation of Alternative Methods joined the other four national validation organizations in the ICATM agreement (55). As ICATM partners, NICEATM and ICCVAM are working with other validation organizations in the evaluation of several non-animal in chemico and in vitro alternatives to the LLNA (56). By communicating and working together, the ICATM validation organizations will identify and embrace scientifically sound and robust test methods that will protect human and animal health and the environment and that will eventually replace the use of animals in ACD hazard testing.

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62 Survey of safety and efficacy information in drug inserts in topical prescription medications*

Danny Zaghi and Howard I. Maibach

INTRODUCTION

Drug inserts are a critical source of information for both health care providers and patients. In many cases, they can be an only source for proper drug application and safety information. Fuchs et al. (1) found that many inserts on drugs, of all possible types (topical, oral, etc.) sold in Europe, missed key safety information including information regarding a daily maximum dose and side effects and were hard to comprehend despite regulation by the European Parliament and the Council of the European Union 2001 requiring these features be present in drug inserts.

To ascertain whether U.S.-sold prescription topical drugs would also be deficient in key safety information as required by a governmental body (in this case the FDA), the following analysis was conducted.

METHODS

Because the purpose of our study is to review safety information in U.S. drugs, we created a set of safety criteria based on current FDA insert safety requirements including indications and usage (what conditions the drug is approved for), contraindications (when not to use the drug), warnings (possible serious side effects), precautions, adverse reactions (all possible side effects observed), overdosage, dosage and administration, and how the drug is supplied. A total of 14 safety criteria were developed (Table 62.1) which we felt provided the most critical safety information for a patient planning on taking a drug. The criteria were designed to be as objective as possible. All evaluations on criteria were made by a second year medical student. Evaluations were made on a "yes" or "no" basis with "yes" indicating that the information was provided (e.g., whether the drug was safe or not safe for nursing women) and "no" implying that no such information was provided with or without a general statement regarding general drug usage such as "because many drugs are excreted in milk, caution should be exercised."

Fifteen topical prescription drugs commonly used in dermatological practice were chosen. An attempt was made to select the most popular drugs used to treat a variety of the most commonly seen dermatological illnesses to provide both a variety and breadth to the drug sample and still be relevant to daily dermatological practice. The drugs used were calcipotriene (calcipotriol) [Dovonex], anthralin (dithranol) [Psoriatec], hydrocortisone probutate (Pandel), ketoconazole (Nizoral), clotrimazole and betamethasone dipropionate (Lotrisone), ciclopirox (Loprox), mupirocin (Centany), benzoyl peroxide (Triaz), clindamycin phosphate (Evoclin), tretinoin (Retin-A Micro), erythromycin (Erygel), fluorouracil (Efudex), permethrin (Acticin), sodium sulfacetamide (Avar), and nystatin (Nystop). The inserts were taken from the Physician Desk Reference online website (2), a database consisting of up-to-date package inserts found in drug packages.

RESULTS

The complete results are listed in Table 62.1.

Results of note included the fact that only one insert (fluorouracil) or 7% of the sample had information regarding the drug's effect on pregnancy in humans. This drug caused severe teratogenic effects when so used in pregnant women. These results are actually somewhat understandable given the difficulty in conducting ethically sound experiments in pregnancy. In light of this, we also evaluated if a certain insert had at least included some information from animal studies on teratogenicity if they had not already included information regarding actual human studies. Ten of the remaining 14 drugs contained at least some animal studies regarding their potential teratogenicity. Four drug inserts (anthralin, benzoyl peroxide, sodium sulfacetamide, nystatin) or 27% of the sample had no information whatsoever on the teratogenicity of the drug being prescribed.

We performed similar evaluations regarding insert information for nursing women and pediatric and geriatric patients. Only two drugs (hydrocortisone probutate, ketoconazole) or 13% contained information regarding use for nursing women. Most of the drugs that did not contain this information contained something along the lines of a general statement that "because many drugs are excreted in milk, caution should be exercised." Unlike the warnings regarding teratogenicity, there were no animal studies present on effects of the drug on lactation in cases when no information on humans was brought.

Only five inserts (hydrocortisone probutate, clotrimazole and betamethasone dipropionate, mupirocin, permethrin, and nystatin)

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TABLE 62.1
Data on Individual Drugs Used

Drug Name	Calcipotriene	Anthralin	Hydrocortisone probutate	Ketoconazole	Clotrimazole and betamethasone dipropionate	Main Chemical	Ciclopirox	mupirocin	benzoyl peroxide	clindamycin phosphate	tretinoin	erythromycin	Main Chemical	Fluorouracil	Permethrin	Sodium Sulfacetamide	Nystatin	Total = 15	%
Name of commercial drug of which drug insert was taken	Dovonex	Psoriatec	Pandel	Nizoral	Lotrisone		Loprox	Centany	Triaz	Evoclin	Retin-A Micro	Erygel		Efudex	Acticin	Avar	Nystop		
Dosage instructions are available	Yes	Yes	Yes	Yes	Yes	Dosage instructions are available	Yes	Yes	Yes	Yes	Yes	Yes	dosage instructions are available	Yes	Yes	Yes	Yes	15	100
The maximum dosage is available	No	No	No	No	Yes	The maximum dosage is available	No	No	No	No	No	Yes	the maximum dosage is available	No	No	No	No	2	13.33333
Information on use for pregnant women	No	No	No	No	No	Information on use for pregnant women	No	No	No	No	No	No	Info on use for pregnant women	Yes	No	No	No	1	6.666667
No information on pregnant human use but brings animal studies on pregnant animals	Yes	No	Yes	Yes	Yes	No information on pregnant human use but brings animal studies on pregnant animals	Yes	Yes	No	Yes	Yes	Yes	No info on pregnant human use but brings animal studies on pregnant animals	No	Yes	No	No	10	66.66667
Information on use for nursing women	No	No	Yes	Yes	No	Information on use for nursing women	No	No	No	No	No	No	Info on use for nursing women	No	No	No	No	2	13.33333
Information on pediatric use	No	No	Yes	No	Yes	Information on pediatric use	No	Yes	No	No	No	No	Info on pediatric use	No	Yes	No	Yes	5	33.33333
Defines age of pediatric	No	No	No	No	Yes	Defines age of pediatric	Yes	Yes	No	Yes	Yes	No	Defines age of pediatric	No	Yes	Yes	No	7	46.66667
Information on geriatric use	Yes	No	No	No	No	Information on geriatric use	No	No	No	No	No	No	Info on geriatric use	No	No	No	No	1	6.666667
Defines age of geriatric	Yes	No	No	No	Yes	Defines age of geriatric	No	No	No	Yes	Yes	No	Defines age of geriatric	No	No	No	No	4	26.66667

(Continued)

TABLE 62.1
Data on Individual Drugs Used *(Continued)*

Drug Name	Calcipotriene	Anthralin	Hydrocortisone probutate	Ketoconazole	Clotrimazole and betamethasone dipropionate	Main Chemical	Ciclopirox	mupirocin	benzoyl peroxide	clindamycin phosphate	tretinoin	erythromycin	Main Chemical	Fluorouracil	Permethrin	Sodium Sulfacetamide	Nystatin	Total = 15	%
Gives information on what patient should do they if they make administrative errors	No	No	No	Yes	No	Gives information on what patient should do they if they make administrative errors	No	No	Yes	Yes	No	No	Gives information on what patient should do they if they make administrative errors	No	Yes	No	No	4	26.66667
Includes information on possible side effects	Yes	Yes	Yes	Yes	Yes	Includes information on possible side effects	Yes	Yes	Yes	Yes	Yes	Yes	Included information on possible side effects	Yes	Yes	Yes	No	14	93.33333
Suitable measures for adverse reaction	Yes	Yes	Yes	Yes	Yes	Suitable measures for adverse reaction	Yes	No	Yes	Yes	Yes	Yes	Suitable measures for adverse reaction	Yes	Yes	Yes	No	13	86.66667
Information on correct storage	Yes	Yes	Yes	Yes	Yes	Information on correct storage	Yes	Yes	Yes	Yes	Yes	Yes	information on correct storage	Yes	Yes	Yes	Yes	15	100
Qualitative statements on the frequency of side effects (rare or common)	No	Yes	No	No	No	Qualitative statements on the frequency of side effects (rare or common)	No	No	No	No	No	No	qualitative statements on the frequency of side effects (rare or common)	Yes	No	Yes	N/A	3	20

or 33% contained information regarding pediatric use. However, seven of the inserts defined what age they consider a pediatric as several inserts with no information regarding pediatric usage still defined the age they consider to be considered a pediatric patient. No animal studies were brought in cases when no information was presented on pediatrics.

Only one insert (calcipotriene) or 7% contained information regarding use by geriatrics. Again no animal studies were brought in any of the cases that did not have information on human geriatrics.

Only four inserts (ketoconazole, benzoyl peroxide, clindamycin phosphate, permethrin) described what a patient should do if they make an administrative error. The recommendations ranged from simply rinsing away the drug from the eye to performing gastric lavage or seeking supportive measures if swallowed. Only two drugs (ketoconazole, permethrin) mentioned what to do if the drug was swallowed.

Three inserts (anthralin, sodium sulfacetamide, fluorouracil), that is, 20% of the sample, contained qualitative statements such as rare or common in describing side effects brought on by the drug.

DISCUSSION

Based on Fuchs's observations, we expected some drug inserts to lack key safety information; however, we did not expect the inserts to be deficient in many key categories of safety information. Particularly concerning was the lack of information on the most commonly seen drug contraindications in pregnancy, nursing mothers, and pediatric or geriatric patients. Since topical drugs in general have a lower bioavailability than oral or systemically administered drugs, the industry might feel that they are less likely to produce the same side effects as other drug types and therefore not devote the same resources to conducting the types of safety tests that they do for other drug types. The problem is that in many cases the patient might also feel the same way about topical drugs and end up taking topical medications more than their prescribed dosage or at times when the drug could actually harm them (e.g., during pregnancy or nursing) because they do not understand how harmful these medications can actually be.

Also of concern was the lack of information on what to do once a drug administration error has been made. In some cases, swallowing the topical medication might only lead to the effects seen when the medication is applied topically and not result in an urgent situation. However, most patients do not recognize that and could feel the need to rush themselves or their children to the emergency room after ingesting a topical medication. On the other hand, swallowing a topical drug in some cases can be dangerous. Providing information on how to respond to an administrative error can help patients determine an appropriate course of action when a mistake is made.

Additionally, several inserts contained qualitative statements such as "rare" or "common" to describe the side effects associated with the drug. Berry et al. (3) found that use of qualitative statements when describing the frequency of side effects led to a gross overestimation of risk by the patient and should thus be avoided whenever possible.

Several significant differences were noted between this study that was only on U.S. topical drugs compared to the Fuchs's study that was on European drugs of all types (oral, intradermal, topical, etc.). Among the Fuchs's study, 87% of the sample included information on pregnant women, whereas only 7% (73%, if animal studies are included) of our sample included such data. Similar findings were present in several of the other potential contraindications we looked at, including pediatrics (Fuchs 64.7% vs. 33% in our sample), geriatrics (Fuchs 25% vs. 7% in our sample), and nursing mothers (Fuchs 86.8% vs. 13% in our sample). Because of the variability and lack of controls between these different studies, the reasons for these discrepancies are unclear but nevertheless of interest.

TABLE 62.2
Possible Ways to Improve Drug Inserts for Topical Drugs

Nonirritating concentration and vehicle for diagnostic patch and photopatch testing
Contact information for dermatologist or other medical source on staff
Topical drug interactions with any skin care products
Stability on skin and after tube or jar opening
Ideal dose in μg/cm^2 as determined from efficacy studies
Index of teratogenicity

Currently, the FDA requires each drug to be identified with a "pregnancy category" with one of five possible categories of A, B, C, D, and X. "A" means that the drug has been proven to be safe to the fetus, whereas at the other end of the spectrum, "X" indicates that the drugs has proved to be harmful to the fetus if taken during pregnancy. "B," "C," and "D" fall somewhere in between that spectrum. Drugs are categorized into these groups based on animal and human studies on the teratogenicity of the drug. Any drug without any teratogenicity information is assigned to group C. Package inserts can be improved by adding similar safety categories for nursing, pediatric, and geriatric patients, which could also be based on existing human and animal studies. This could better help the physician and patient understand the potential risk versus benefits when deciding whether to take a drug.

Inserts for topical drugs can be improved by better customizing them to deal with the unique features encountered when applying a drug to the skin (Table 62.2). A section can be included in each insert, which details the drug's interactions, if any, with any commonly used skin care products or any cross reactions that produce allergic or photoallergic dermatitis. This would allow the physician and patient to adjust the prescribing regimen and prepare for any adverse reactions. The insert could also include information on a drug's stability after tube or jar opening as well as an ideal dose in ug/cm^2 based on efficacy studies.

Another simple way the industry could improve drug inserts would be by providing contact information for a dermatologist on staff. In such a scenario, patients and physicians would have access to a source with medical knowledge and experience with the drug who could answer their questions about a product or field reports of new adverse reactions.

CONCLUSION

While it might not be feasible for a pharmaceutical company to have thoroughly studied all relevant safety information on a new drug before its initial release, there must still be an active initiative upon the company's part to investigate the effects of the drug, especially against the most likely contraindicated populations of pregnant, nursing, and pediatric and geriatric patients, even after

its release. Even if the information is only from animal studies, it could still play a role in a patient's decision-making regarding whether to use a drug. Additionally, information on what to do following incorrect usage of a topical medication, though it might seem academic to physicians and pharmacists, is critical information for the patient and should be included in all package inserts for topical medications.

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63 Lack of drug interaction conformity in commonly used drug compendia for selected at-risk dermatologic drugs*

Katherine Willard, Ivy Lee-Keltner, Stephanie Chao, and Howard I. Maibach

INTRODUCTION

As medical pharmacology has expanded over the past several decades, so has the knowledge that drug interactions not only exist, but can also pose a serious threat to the health of many patients each year. Extrapolating data from the Medical Practice Study (MPS), it has been estimated that over a million patients suffer iatrogenic injury in hospitals across the country each year (1). About 19.4% of the medical injuries identified in the MPS were due to the use of drugs (2). A separate study has shown that specifically, drug–drug interactions represent up to 4% of preventable in-hospital adverse drug events (3). In the outpatient realm, it has further been demonstrated that drug–drug interactions are an important contributor to numerous emergency room visits each year. Amongst all adverse drug events prompting emergency room visits, patients with drug–drug interactions often have severe reactions and require hospitalization (4).

To avoid iatrogenic harm due to drug–drug interactions, many physicians rely on a variety of drug compendium when making clinical decisions regarding the prescribing of medications. Unfortunately, information provided about drug interactions is often inconsistent among various compendia. The following chapter serves to highlight the inconsistencies among four U.S. drug compendia for four systemic medications frequently prescribed in the field of dermatology. (5)

Four systemic medications commonly prescribed in the field of dermatology were selected. They were as follows: dapsone, erythromycin, methotrexate, and prednisone. Drug interaction information for these medications was obtained from four widely used English language drug references: Physicians' Desk Reference (PDR) (6,7), American Hospital Formulary Service (AHFS) Drug Information (2003), (8) U.S. Pharmacopeia Drug Information (USP DI) [21st edition], (9) and Mosby's GenRx (11th edition, 2001) (10).

From these four sources, drug interactions were compiled for each of the selected medications (Tables 63.1, 63.2, 63.3, 63.4, 63.5, adapted from Chao et al. (5)). In compiling these tables, all drugs mentioned in the respective "Warnings," "Precautions," "Laboratory Values Alterations," or "Drug Interactions" sections of each monograph were included. If a reference listed a drug class, only drugs specifically listed in the "Drug Interactions" sections were included. However, if drug classes were listed but the text referred to appendices for specific listings of agents within those classes, only the drug classes were included. From the data summarized in Tables 63.1, 63.2, 63.3, 63.4, percentages of drug–drug interaction agreement across the four sources were calculated. These are summarized in Table 63.5, excerpted from Chao et al. (5).

DAPSONE

GenRx and PDR present the most limited lists of drug interactions for dapsone; however, they list the same three interactions: folic acid antagonist (pyrimethamine), Rifampin (rifampicin), and trimethoprim or triamterene. In contrast, USP DI lists 6 interactions and does not include pyrimethamine/folic acid antagonists. AHFS lists a total of 15 interactions. Sixteen total interactions were listed at least once in one of the four sources, with most comprehensive (AHFS) listing 93.8% of these.

ERYTHROMYCIN

37 total interactions were listed across all sources for erythromycin. As was observed in the case of dapsone, GenRx and PDR list the same interactions for erythromycin (a total of 20 in these two compendia). The most comprehensive source, AHFS, listed 70.3% of the total amount of drug interactions found.

METHOTREXATE

Forty-five total drug interactions were listed for methotrexate in one of the four sources. The most comprehensive of these, USP DI, listed 26 of the 45 (57.8%). Only 4 (8.9%) interactions were listed in all four sources. These were folic acid, salicylates, sulfonamides, and NSAIDs; however, each source listed different specific drugs in the class "NSAIDs."

PREDNISONE

The number of drug interactions documented for prednisone varied widely. This figure ranged from 8 interactions noted by GenRx to 51 interactions noted by USP DI. No two sources had complete agreement. There were four medications or medication classes listed in all four sources ("anticoagulants," "phenobarbital [phenobarbitone]," "barbiturates," "phenytoin," and "rifampin [rifampicin]").

*This chapter is adapted from Chao SD, Maibach HI. *Lack of drug interaction conformity in commonly used drug compendia for selected at-risk dermatologic drugs*. Am J Clin Dermatol 2005; 6: 105–11, with permission.

TABLE 63.1
Interacting Drugs with Dapsone

Interacting Drug	Drug Compendium USPDI	GenRx	PDR	AHFS
Didanosine	•			•
Clofazimine				•
Aminobenzoates	•			
Blood Dyscrasia-Causing Medications[a]	•			•
Folic Acid Antagonist (Pyrimethamine)		•	•	•
Hemolytics	•			•
Nitrite				•
Aniline				•
Phenylhydrazine				•
Naphthalene				•
Niridazole				•
Nitrofurantoin				•
Primaquine				•
Rifampin	•	•	•	•
Trimethoprim or Triamterene	•	•	•	•
Probenecid				•

[a]For Tables I–IV, drug classes/groupings (in bold) reflect classifications used by the compendia. Drugs that appeared in overlapping classifications were listed above under only one classification. Drugs listed in compendia appendices were not included. *Source*: From Ref. (5).

TABLE 63.2
Interacting Drugs with Erythromycin

Interacting Drug	Drug Compendium USPDI	GenRx	PDR	AHFS
Alcohol	•			
Anticoagulants		•	•	
Warfarin	•			•
Chloramphenicol	•			•
Cisapride		•	•	•
Clindamycin				•
Clozapine				•
Cytochrome P450 metabolized drugs		•	•	•
Alfentanil	•	•	•	•
Astemizole	•	•	•	•
Bromocriptine		•	•	•
Carbamazepine	•	•	•	•
Cyclosporine	•	•	•	•
Disopyramide		•	•	•
Hexobarbital		•	•	•
Phenytoin		•	•	•
Tacrolimus		•	•	
Terfenadine	•	•	•	•
Valproate/Valproic Acid	•	•	•	
Digoxin	•	•	•	•
Ergotamine or dihydroergotamine	•	•	•	•
Hepatotoxic Medications	•			
Lovastatin	•	•	•	•
Lincomycin	•			
Midazolam	•	•	•	•
Ototoxic Medications	•			
Penicillins	•			•
Quinidine				•

(Continued)

TABLE 63.2
Interacting Drugs with Erythromycin *(Continued)*

Interacting Drug	Drug Compendium USPDI	GenRx	PDR	AHFS
Rifampin				•
Streptomycin				•
Sulfonamides				•
Triazolam	•	•	•	•
Xanthines	•			
Aminophylline	•			
Caffeine	•			
Oxtriphylline	•			
Theophylline	•	•	•	•

Source: From Ref. (5).

TABLE 63.3
Interacting Drugs with Methotrexate

Interacting Drug	Drug Compendium USP DI	GenRx	PDR	AHFS
Alcohol	•			
Anticoagulants	•			
Coumarin-derivative	•			
Indanedione-derivative	•			
Blood Dyscrasia Agents	•			
Bone Marrow Depressants	•			
Radiation therapy	•			
Sulfamethoxazole		•	•	
Co-trimoxazole				•
Folic acid	•	•	•	•
Hepatotoxic medications	•		•	•
Azathioprine			•	•
Retinoids (Etretinate)			•	•
Sulfasalazine			•	•
Leuvocorin		•	•	
Neomycin (p.o.)	•			
Nonabsorbable Broad Spectrum Antibiotics		•	•	
NSAIDS	•	•	•	•
Diclofenac	•			
Indomethacin	•			•
Ketoprofen				•
Naproxen	•			
Phenylbutazone	•			
Penicillins	•		•	•
Amoxicillin				•
Carbenicillin				•
Mezlocillin				•
Protein-Bound Drugs & Weak Organic Acids	•			•
Aminobenzoic Acid				•
Chloramphenicol		•	•	•
Phenytoin		•	•	•
Phenylbutazone		•	•	•
Salicylates	•	•	•	•
Sulfonamides	•	•	•	•
Sulfonylureas				•
Tetracycline		•	•	•
Pyrimethamine	•			•

(Continued)

TABLE 63.3
Interacting Drugs with Methotrexate *(Continued)*

	Drug Compendium			
Interacting Drug	**USP DI**	**GenRx**	**PDR**	**AHFS**
Renal Toxic		•		
Cisplatin		•		
Probenecid	•	•	•	
Theophylline	•		•	•
Triamterene or Trimethoprim	•	•	•	
Vaccine	•			•
w/ live virus	•			•
w/ killed virus	•			

Source: From Ref. 5.

TABLE 63.4
Interacting Drugs with Prednisone

	Drug Compendium			
Interacting Drug	**USPDI**	**GenRx**	**PDR**	**AHFS**
Alcohol	•			
Acetaminophen	•			
Aminoglutethimide	•			
Amphotericin B	•			
Carbonic Anhydrase Inhibitors (Acetazolamide)	•			
Anabolic Steroids or Androgens	•			
Antacids	•			
Anticholinergics	•			
Anticholinesterase				•
Ambenonium				•
Neostigmine				•
Pyridostigmine				•
Anticoagulants	•	•	•	•
Doumarin	•			
Heparin	•			
Indanedione-Derivative	•			
Streptokinase	•			
Urokinase	•			
Antidepressants – Tricyclic	•			
Antidiabetic Agents – Oral or Insulin	•			•
Antithyroid Agents or Thyroid Hormones	•			
Asparaginase	•			
Oral contraceptives, Estrogens	•			•
Benzodiazepines	•			
Cyclosporine	•		•	•
Cyproheptadine	•			
Digitalis glycosides	•			
Diuretics	•			
Folic Acid	•			
Glutethimide	•			
Hepatic Enzyme Inducing Agents (P450 Isoenzyme 3A4)	•	•		•
Ephedrine	•			•
Phenobarbital, Barbiturates	•	•	•	•
Phenytoin	•	•	•	•
Rifampin	•	•	•	•
Immunosuppressants	•			
Indomethacin	•			
Iophendylate	•			
Isoniazid	•			
Ketoconazole		•	•	
Meprobamate	•			
Methaqualone	•			
Methyprylon	•			
Metrizamide	•			
Mexiletine	•			
Mitotane	•			
Neuromuscular Blocking Agents (non-depolaring)	•			
NSAIDs	•			•
Potassium Depleting Drugs				•
Amphotericin B				•
Ethacrynic Acid				•
Furosemide				•
Thiazides				•
Potassium Supplements	•			
Ritodrine	•			
Salicylates	•	•	•	•
Sodium Containing Drugs or Foods	•			
Somatrem or somatropin	•			
Streptozocin	•			
Troleadnomycin	•	•	•	
Vaccines, Live Virus, or Other Immunizations	•		•	•

(Continued)

TABLE 63.4
Interacting Drugs with Prednisone *(Continued)*

Source: Reproduced from Ref. 5 with permission from Adis, a Wolters Kluwer business (© Adis Data Information BV 2005. All rights reserved.

TABLE 63.5
Summary of Agreement of Drug-Interactions Among Compendia

		Sources Listing Drug–Drug Interactions[a]			
Study Drug	**Total Number of Interactions**	**1**	**2**	**3**	**4**
Dapsone	16	93.8%	37.5%	18.8%	12.5%
Erythromycin	37	67.6%	62.1%	48.6%	29.7%
Methotrexate	45	57.8%	53.3%	28.9%	8.9%
Prednisone	61	83.6%	23.0%	13.1%	8.2%

[a]In the case of only one source, percentages were calculated based on the most comprehensive source.
Source: Reproduced from Ref. 5.

CONCLUSION

As one can see, considerable discrepancies among the references were consistently demonstrated for each of the four medications examined here. There are many possible reasons for these observed discrepancies. Notably, the criteria for inclusion and exclusion of particular interactions are not clearly defined by the texts. Furthermore, none of the compendia surveyed provided quantitative data to demonstrate the strength of evidence for each interaction listed. The texts also did not consistently provide references for each monograph. Assessing clinical relevance of the interactions listed is made more challenging by

the fact that these sources fail to make clear the severity of the interactions listed. Each source does document that each drug interaction listed is "of clinical significance," however, only the USP DI indicated which interactions were of "major clinical significance."

These wide variations suggest the need for resolution, and for a comprehensive source documenting drug–drug interactions and the severity of these interactions. The discrepancies between these texts place physicians relying on a single reference at increased risk for prescribing medications that have the potential to elicit significant drug–drug interactions in their patients. This data demonstrates a clear need for a compendium of drug monographs with a more comprehensive listing of drug–drug interactions, as well as explanations of the severity of the reactions and a summary of the available evidence supporting each interaction.

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64 OECD guidelines for testing of chemicals

Klaus-Peter Wilhelm and Howard I. Maibach

PREFACE

General

This publication contains an excerpt of the official OECD Guidelines for Testing of Chemicals as adopted by the OECD Council. The Test Guidelines have been developed initially under the OECD Chemicals Testing Programme (see below), and subsequently, since 1981, as provided by the council under the OECD Updating Programme for Test Guidelines.

Whenever testing of chemicals is contemplated, the OECD Test Guidelines should be consulted. Since the Test Guidelines have been endorsed by the OECD member countries, their use in the generation of data provides a common basis for the acceptance of data internationally, together with the opportunity to reduce direct and indirect costs to governments and industries associated with testing and assessment of chemicals.

Other methods and guidelines not included in this publication may be judged to be appropriate in testing chemicals in certain scientific, legal, and administrative contexts.

The OECD Council Decision on Mutual Acceptance of Data (12th May 1981; C[81]30) affirms that data generated in one country in accordance with the OECD Test Guidelines – and additionally in accordance with the OECD Principles of Good Laboratory Practice – should be accepted in OECD countries for purposes of assessment and other uses relating to protection of man and the environment. The full text of this Decision and the OECD Principles of Good Laboratory Practice may be found in the Appendix to the OECD Guidelines for Testing of Chemicals.

The OECD Test Guidelines contain generally formulated procedures for the laboratory testing of a property or effect deemed important for the evaluation of health and environment hazards of a chemical. The Guidelines vary somewhat in respect of detail, but include all the essential elements which, assuming good laboratory practice, should enable an operator to carry out the required test.

OECD Test Guidelines are not designed to serve as rigid test protocols. They are instead designed to allow flexibility for expert judgment and adjustments to new developments. It is intended that the OECD Test Guidelines be used by experienced laboratory staff familiar with the type(s) of testing involved. Proper conduct of testing and associated interpretation of results can only be achieved by appropriately trained personnel with access to equipped laboratory facilities. The loose-leaf system chosen for the Guidelines allows for additions and changes to be made when necessary. Information will be circulated when such changes occur resulting from work under the Updating Programme.

OECD Chemicals Testing Programme

The OECD Chemicals Testing Programme was launched by the Chemicals Group in November, 1977. It comprised six Expert Groups under the leadership of individual member countries. One of these groups, the Step System Group, worked on phased approaches to testing and assessment of chemicals (see below).

Five of the groups reviewed the state-of-the-art of methods and produced draft Test Guidelines. The following areas were covered:

i. Physical–chemical properties (Lead country – Germany)
ii. Effects on biotic systems other than man (Lead country – the Netherlands)
iii. Degradation/accumulation (Lead countries – Japan/Germany)
iv. Long-term health effects (Lead country – the United States)
v. Short-term health effects (Lead country – the United Kingdom)

Some 300 experts, drawn from academia, government, industry, international organizations, and other sectors, took part in the Programme. In all, about 50 meetings were held during 1978–1979 under the auspices of the OECD Chemicals Testing Programme.

To improve the international validation of tests, several methods were subjected to laboratory intercomparison exercises in the Chemicals Testing Programme. This work is being continued under the OECD Updating Programme.

In December 1979, the five Expert Groups working on test methods submitted their reports to the OECD. The two groups on health effects submitted a combined report. The reports contained draft Test Guidelines and an analysis of approaches to testing within the respective areas. During 1980, the draft Test Guidelines were subjected to an extensive commentary and review process. Member countries were invited to submit comments to the OECD, which were subsequently taken into account by a Review Panel, established to finalize the product for adoption and printing. The Panel worked in close collaboration with the Chairman of the Expert Groups. The review process was concluded by the Chemicals Group and the Environment Committee of the organization, which endorsed these Test Guidelines prior to their formal submission to the OECD Council.

The subject areas covered by the Expert Groups under the Chemicals Testing Programme have largely been kept separate in this publication. Thus, OECD Test Guidelines are presented under four different sections:

- Physiochemical properties
- Effects on biotic systems other than man

- Degradation/accumulation
- Health effects

Each section is preceded by a summary of considerations raised in the individual expert group reports. These summaries reflect some of the major observations and explanations made at the scientific level during the preparatory process. Further, major portions of the expert group reports have been absorbed into the on-going activities of OECD on chemicals.

OECD Updating Programme for Test Guidelines

In 1981, the OECD Updating Programme for Test Guidelines was established by Member countries in consultation with the Commission of the European Communities. The aim was to ensure that OECD Test Guidelines will not become outdated as a result of major changes in the state-of-the-art or scientific advances.

The Updating Programme is considering:

a. Proposals for new or modified tests that offer conspicuous advantages over those already adopted.
b. New guidelines which are being developed in areas not yet covered.
c. Incorporation of results from the Chemicals Testing Programme into OECD Test Guidelines.
d. Those matters which need further investigation and research.

OECD Principles for Testing and Assessment of Chemicals

The OECD Test Guidelines are but one component in an OECD strategy to make testing of chemicals more systematic, relevant, and cost-effective within an international framework, which could lead to increased exchange and acceptance of test data between countries. This strategy has been developed with vigor in the organization during the 1970s, leaving several important questions yet to be resolved.

While the OECD Test Guidelines can properly be used in establishing one effect or property, the Guidelines were developed under programmes directed toward an integrated and comprehensive approach to testing and assessment. Thus, the OECD Council, in 1974 and 1977, developed recommendations, which deal respectively with “The Assessment of the Potential Environmental Effects of Chemicals” (C[74]215) and "Guidelines in Respect to Procedures and Requirements for Anticipating the Effects of Chemicals on Man and in the Environment" (C[77]97[Final]).

In 1974, the OECD Council recommended that prior to marketing of chemicals, their potential effects on man and his environment should be assessed.

This concern, that assessments should encompass both man and his environment, was reflected in the subject areas chosen for the OECD Chemicals Testing Programme, and is also reflected in the disposition of the Test Guidelines into sections.

Some outstanding features with respect to testing and assessment which derive from the 1977 OECD Council Recommendations can be summarized as follows:

i. Chemical substances – with special emphasis on new substances – should be subjected to systematic assessment for potential effects, in relation to both human and environmental hazard.
ii. It is possible to determine no more than the likelihood of adverse affects from chemicals, and this can only be done through the application of expert judgment based on methods that are technically practicable, as well as economically acceptable.
iii. Responsibility for generating and assessing the data necessary to determine the potential effects of chemicals must be part of the overall function and liability of industry.
iv. A phased approach should be applied in data gathering and assessments.

These four principles also provided guidance to the Expert Groups in their work in the Chemicals Testing Programme.

The need for expert judgment in testing and assessment has been emphasized throughout the work on chemicals in OECD. The Expert Groups under the OECD Chemicals Testing Programme reaffirmed this need when they selected methods that were regarded as technically practicable and economically acceptable for inclusion into OECD Test Guidelines.

The question of a phased approach to testing and assessment is an important concept that is under continuous active consideration in OECD within the Chemicals Testing Programme. All the Expert Groups have contributed to the framework of an overall scheme for testing and assessment of chemicals.

In their work the five Expert Groups on test methods identified steps in which testing and assessment might proceed. The early steps were usually simple in character with the objective of establishing a first indication of hazard. Further steps brought the testing and assessment into a sophisticated and time-consuming range of tests, characterized by increased confidence in the assessments.

The Steps Systems Group, the sixth Expert Group established under the Chemicals Testing Programme, draws upon the work of the other Expert Groups and is currently developing an integrated stepwise approach to testing and assessment of chemical hazard to man and his environment.

An important outcome of the work of the Step Systems Group is the OECD Minimum Pre-marketing set of Data (MPD). MPD lists some 35 individual data components that normally would be sufficient to perform a meaningful first assessment of the potential hazard of a chemical.

Finally, it should be recognized that elaboration of principles for testing and assessment of chemicals is a continuing process within OECD. This process has been, and remains, possible only through the generous provision of time, knowledge, and enthusiasm from the participating experts, and the active support of Member countries.

ADOPTED TEST GUIDELINES

The complete list of OECD guidelines is available on the OECD webpage (www.oecd.org). Following is a list of those guidelines pertaining to dermal and ocular toxicity.

402 Acute Dermal Toxicity (Updated Guideline, adopted 24th February 1987)
404 Acute Dermal Irritation/Corrosion (Updated Guideline, adopted 24th April 2002)
405 Acute Eye Irritation/Corrosion (Updated Guideline, adopted 24th April 2002)

406 Skin Sensitization (Updated Guideline, adopted 17th July 1992)
410 Repeated Dose Dermal Toxicity: 21/28-day Study (Original Guideline, adopted 12th May 1981)
411 Subchronic Dermal Toxicity: 90-day Study (Original Guideline, adopted 12th May 1981)
427 Skin Absorption: In Vivo Method (Original Guideline, adopted 13th April 2004)
428 Skin Absorption: In Vitro Method (Original Guideline, adopted 13th April 2004)
429 Skin Sensitization: Local Lymph Node Assay (Updated Guideline, adopted 23rd July 2010
430 In Vitro Skin Corrosion: Transcutaneous Electrical Resistance Test (TER) (Original Guideline, adopted 13th April 2004)
431 In Vitro Skin Corrosion: Human Skin Model Test (Original Guideline, adopted 13th April 2004)
432 In Vitro 3T3 NRU Phototoxicity Test (Original Guideline, adopted 13th April 2004)
435 In Vitro Membrane Barrier Test Method for Skin Corrosion (Original Guideline, adopted 19th July 2006)
438 Isolated Chicken Eye Test Method for Identifying Ocular Corrosives and Severe Irritants (Original Guideline, adopted 7th September 2009)
439 In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method (Original Guideline, adopted 22nd July 2010)
442 A Skin Sensitization: Local Lymph Node Assay: DA (Updated Guideline, adopted 22nd July 2010)
442B Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA (Updated Guideline, adopted 22nd July 2010)
451 Carcinogenicity Studies (Updated Guideline, adopted 7th September 2009)

DRAFT TEST GUIDELINES FOR WHICH COMMENTING PERIOD HAS EXPIRED AND WHICH ARE BEING REVISED OR FINALIZED

434 Acute Dermal Toxicity-Fixed Dose Procedures
Draft New Guideline (May 2004)
Deadline for public comments passed: 16 July 2004

65 Dermatologic drugs withdrawn by the FDA for safety reasons

Melissa Martin and Howard Maibach

INTRODUCTION

The beginning of dermatoxicology can be dated to 1944, when the late John Draize introduced the Draize tests (1). A special issue of Toxicology and Applied Pharmacology has already reviewed some of the advances in this field (2). We take the liberty here of reviewing dermatoxicologic drug related withdrawals in light of their history and the current knowledge of the underpinnings of the science and art of dermatoxicity as it relates to the adoption of new dermatologically oriented drugs. We focus on FDA withdrawals starting with the Kefauver Harris Amendment of 1962, which required drug sponsors to provide safety and effectiveness data before approval (3). Note that nonclinical and clinical evaluation of drugs has changed dramatically in the past 15 years–following increased dermatoxicologic knowledge.

BITHIONOL

Bithionol, used in cosmetic ingredients as an antibacterial agent, was found in products, including creams, lotions, shampoo, and acne preparations. Its photocontact sensitization effect became evident after clinical cases appeared along with positive photopatch tests (4). Persistent light reaction could persist long after exposure to the antibacterial agent had ended. Cross-sensitization to halogenated salicylanilides and hexachlorophene also occurred (4). Bithionol was more potent as a sensitizer than dibromosalicylanilide (DBS) and tribromosalicylanilide (TBS), but less potent than terachlorosalicylanilide (TCSA), and its maximum sensitivity occurred at a wavelength of 360 nm (5).

The FDA proposed the ruling to withdraw products containing bithionol on July 19, 1967 (6). Three months later, the proposal went into law. New drug applications (NDAs) for any product containing bithionol were revoked. Soon after the FDA's proposal, the manufactures of the following products withdrew their products (Table 65.1) (6).

According to the FDA report, there was no evidence of a photosensitization effect when these products were approved and the methods of available tests were not "deemed reasonably applicable when such applications were approved" (6). However, by April 1963, there was documentation that bithionol did not normally produce positive patch tests, but that it did produce positive and eczematous photopatch tests (7). The photosensitivity effect went beyond the sunburn spectrum, including longer wavelengths of light, which caused patients to experience reactions through glass windows (7).

We do not know if the Draize Repeat Insult Patch Tests had been performed before bithionol approval. If positive, as in the case of 3,3',4,5'- TCSA), this might have provided a hint of the need for further risk assessment. Predictive photoallergy assays had not yet been developed.

HALOGENATED SALICYLANILIDES: 3, 3', 4', 5-TCSA, TRIBROMSALAN, DIBROMSALAN, AND METABROMSALAN

The following antimicrobial halogenated salicylanilides were used as ingredients in many products: tribromsalan (TBS, 3,4',5-triromosalicylanilide), dibromsalan (DBS, 4',5-dibromosalicylanilide), metabromsalan (MBS, 3,5-dibromosalicylanilide), and 3,3',4,5'-TCSA. They were largely used in antibacterial soaps and as antimicrobials in cosmetic products to reduce skin bacteria. However, they caused photoallergic contact dermatitis and some were also cross-sensitizers (8). Some affected individuals continued to experience symptoms months and years after no longer being exposed to the product (persistent light reaction) (8). Patients experiencing photosensitization had dermatitis in sun-exposed areas of the body, including the face, neck and dorsal area of the hands.

On October 30, 1975 the FDA announced that products containing any of these halogenated salicylanilides was considered a new drug or an adulterated cosmetic (9). When the FDA proposed this ruling on September 19, 1974, it confirmed that none of the currently approved NDAs for prescription or over the counter (OTC) drugs contained halogenated salicylanilides (10). These products had already been withdrawn from the market or their manufacturers had replaced the unsafe compound with another substance. The FDA determined that the risks of using products with halogenated salicylanilides outweighed their benefits, and that safer alternatives were available. Based on this data, the FDA recommended that halogenated salicylanilide-containing products must have that compound removed. Note that the FDA was only supposed to review TBS, but extended its ruling to more potent halogenated salicylanilides, such as DBS, MBS, and TCSA (10).

Halogenated salicylanidlides had been a health concern since the early 1960s. Daryl Wilkinson, a British dermatologist, had several cases of photodermatitis in 1960 during the "epidemic," including 29 workers from a single factory (11). These workers had been using a germicidal soap containing TCSA that had already been withdrawn by its manufacturers. TCSA was withdrawn from the market in England soon after these cases arose.

TABLE 65.1
Withdrawn Products Containing Bithionol

Manufacturer	Product Name
Rexall Drug Company	Rexall Medicated Dusting Powder
Medical Arts Supply Company	Surginol Surgical Soap
Shulton, Inc.	Thylox Sulfur Cream
Shulton, Inc.	Thylox Sulfur Soap
Huntington Laboratories	Degerm with Actamer
West Chemical Products	Lan-O-Kleen
North Coast Chemical Company	Coco-Borax Powered Hand Soap
Armour Grocery Products Division of Armour & Co.	Dial Deodorant Soap
Purex Corporation	Cutitone Acne Cream

During this time, only few cases of photodermatitis were reported in the U.S., which were caused by soaps containing TBS. There was also evidence that photocross-sensitization occurred with TCSA and TBS, and that 3200–4500 angstrom units of UVA were required for a reaction (12).

By 1975, the FDA had found TBS to be a primary photosensitizer as supported by 80 cases in Denmark during the late 1960s (13). All of the patients had exposure to a soap containing TBS. TCSA, the suspected primary photosensitizer in this case, had never been on the market in Denmark and therefore there was no reason to believe this was simply a cross-sensitization reaction. Masuda et al. (1971) also provided evidence that TBS was a primary sensitizer (13). However, guinea pigs tested negative for TBS photosensitization, and critics argued the FDA had made an incorrect decision in banning TBS from products (13). The FDA responded by saying that the guinea pig model was not 100% equivalent to humans and differences in sensitization were expected. A study published in 1966 also found DBS to be a photosensitizer (14).

The FDA noted that once dermatologists became aware of the photosensitization caused by halogenated salicylanilides in soaps they stopped reporting these cases and instructed their patients to discontinue using the product (13). The FDA waited several years after clinical cases were reported to officially ban halogenated salicylanilides in products. The reasons for this remain unknown.

In retrospect, techniques in use during this time Repeat Insult Patch Test (RIPT) would have identified at least TCSA as a potent contact sensitizer (15), even though photosensitization assay had not yet been developed. It remains to be seen whether our current photosensitization assays will prevent further epidemics with new agents.

CHLOROFORM

Chloroform was found in numerous products, including cosmetics, cough and cold preparations, topical liniments, and toothpaste. It was also once used as an anesthetic and in an experimental treatment of herpes zoster (16). Chloroform can be absorbed through three distinct routes: gastrointestinal, respiratory, and dermal (17). It is a volatile liquid first produced in 1903, and by 1974 its production in the U.S. reached 300 million pounds (18). However, 96% of the chloroform produced was used to make fluorocarbons (16).

On March 1, 1976, the National Cancer Institute reported that giving oral chloroform to mice and rats resulted in the development of hepatocellular carcinoma and renal tumors, respectively (18). By July 29, 1976 the FDA stated that any product containing chloroform would be considered a new drug (17). Products with residual amounts of chloroform due to use as a processing solvent or as a byproduct were exempted from this ruling. At this time, the FDA also required all manufactures with an approved NDA for a chloroform-containing product to submit a revised supplemental application, which led the manufacturer to replace chloroform with another substance (17). These FDA guidelines also made it mandatory for these same manufactures to stop marketing their product until further notice if their product contained more than 1% chloroform. Continuation of product marketing was dependent on replacing the chloroform with another substance and receiving written approval from the FDA. For products that contained less than 1% chloroform, manufactures only had to submit a supplemental application specifying the substance that would replace chloroform, but they did not have to wait for written approval from the FDA to continue product marketing.

Since chloroform was a carcinogen in experimental animal studies, (16) it was possibly a human carcinogen and this explains the FDA's actions. However, at the time, chloroform did not have an association with cancer in humans. Today, it is regarded as a reasonably anticipated human carcinogen, since studies have failed to provide a cause-and-effect relationship (19). Studies have only provided associations between chloroform and cancer of the gastrointestinal tract and bladder in humans (16).

Taken together, experimental carcinogenicity studies of every chemical with significant human exposure were not conceived when chloroform was first introduced in the early 1900s and today the clinical relevance of positive in vitro and animal carcinogenicity studies are far from fully understood. Readers are referred to the International Agency for Research on Cancer (IARC) monographs for what known of the clinical relevance of the in vitro and in vivo carcinogenicity assay in animals (20).

AZARABINE (TRIAZURE TABLETS)

Azarabine, indicated for psoriasis and also used for mycosis fungocides and rheumatoid arthritis, was manufactured by Calbiochem. The drug was withdrawn on June 10, 1977 due to severe thromboembolic events (21). Azarabine was administered orally and converted to 6-azaurdins 5'-monophsophate, which inhibited orotidylate decarboxylase (22). This inhibition prevented the conversion of orotidylic acid to uridylic acid and slowed down the de novo pyrimidine pathway.

Thromboemboli events were witnessed during the premarketing phase (23). The drug was approved on January 1, 1975 as patients with psoriasis were considered to be at higher risk for thromobotic events. In one study, 36 patients were treated with azaribine from 1972 to 1974 (24). A positive outcome was found in 78% of patients. Patients took 200 or 125 mg/kg/day of the drug and a better outcome did not depend on dose. Side effects, however were dose dependent (Table 65.2).

Although central nervous system effects were just as common in the 125 mg as in the 200 mg group, the latter group experienced more severe symptoms, including lethargy, loss of memory, diplopia, expressive aphasia, and coma. Two patients experienced

TABLE 65.2
Azarabine Dose-Dependent Side Effects

Side Effect	125 mg/kg/day	200 mg/kg/day
Anemia	40%	10%
Leukopenia	8%	~0
Gastrointestinal disturbances	35%	10%

TABLE 65.3
Thrombotic Events Associated with Azarbine

Thrombotic Event	Number of Patients Affected
Thrombophlebitis	7
Mesenteric thrombosis	2
Pulmonary embolism	1
Myocardial infarction	5
Arterial occlusion	3

thrombophlebitis and one patient experienced axillary thrombosis. However, of the 299 patients in the U.S. taking 125 mg/kg of azarabine, nine experienced a thrombotic event. A more complete summary of thrombotic events is offered when the 600 patients taking azarabine are considered (Table 65.3).

These vascular problems occurred in 3% of the 600 patients studied. The drug was removed 2.4 years after approval (25) and continued reports of thrombotic events, including thrombosis of a digital artery.

In retrospect, observations made in the preapproval phase might have been a signal to add more patients in phase 3 observations.

ZIRCONIUM AEROSAL PRODUCTS

Zirconium aerosol products were pulled from the market for safety reasons. These products were available OTC and used as antiperspirants.

After a review by the OTC Antiperspirant Panel, the Commissioner of Food and Drugs proposed on June 5, 1975 that aerosol products containing zirconium would be considered a new drug or cosmetic (26). Zirconium aerosol antiperspirants were not more effective than non-aerosolized antiperspirants–the safer alternative–and their benefits did not outweigh their risks. As Category II, they were not generally recognized as safe (GRAS) (26). They were implicated in granuloma formation in human skin and studies using animal models illustrated they were toxic to organs, including the lungs (27). The FDA invited comments on the proposed ruling.

At the time of the proposal, the Commissioner of Food and Drugs stated that "because self-medication is essential to the nation's health care system, it is imperative that over-the-counter drugs be safe, effective, and adequately labeled. He further stated, 'FDA accepts as necessary and desirable the tradition of self-medication. The consumer in turn has every right to expect that the OTC drugs he buys are safe and well labeled, and that they will perform as the manufacturer claims'" (26). Zirconium aerosol products could not meet consumer expectations with certainty.

Zirconium compounds, specifically zirconium lactates, were first used in antiperspirants in the 1950s (26). Users developed papules in the axillae that histological resembled granulomas as seen in sarcoidosis. Subsequently, zirconium oxide was used for poison ivy dermatitis and it caused the development of papules also considered to be granulomas (26). With zirconium aerosol antiperspirants, particles could be inhaled and cause the formation of granulomas in the lungs, which are more difficult to detect (28).

By May 1976, the manufactures of these products had stopped production (29). The final ruling from the FDA came two years later after the initial proposal. On August 16, 1977, the FDA officially stated that any aerosol or cosmetic containing zirconium requires a new drug application (29). This would ensure that each product would undergo safety testing before being available to consumers. By September 15, 1977, zirconium-containing aerosols were no longer available in the U.S. (29). However, aluminum zirconium antiperspirants are currently on the market OTC.

In retrospect, the models proposed by the late William Epstein might predict such an event with new formulations (30).

Potassium arsenite (Fowler's Solution)

All products containing potassium arsenite were withdrawn from the market in 1980. Fowler's Solution, containing 10% potassium arsenite, had been used for decades for a variety of medical conditions, including those involving the skin. It was available to physicians and their patients prior to the establishment of the NDA process in 1938 (31).

In 1806, Thomas Girdlestone reported the use of arsenite for skin disorders in "Observations on the Effects of Dr. Fowler's Mineral Solution in Lepra and other Diseases"(32). The publication described potassium arsenite as having positive outcomes for lepra, prurigo, and psoriasis.

The link between potassium arsenite and cancer had been observed for over a century before product withdrawal. In the late nineteenth and early twentieth century, Dr. Jonathan Hutchinson, a British surgeon, observed that several of his patients who had been treated with potassium arsenite for psoriasis, had developed skin disorders, including arsenic keratosis and squamous cell carcinoma (33). He also noted that these lesions spared psoriatic plaques. Cancer of the lung and bladder, and angiocarcinoma of the liver were also associated with potassium arsenite ingestion (34). The product was later established to be both toxic and a carcinogen. The FDA determined it was a new drug in 1980, and soon after its manufacturers withdrew it. However, the International Agency for Research on Cancer (IARC) had conducted studies exposing mice to potassium arsenite, and no carcinogenic effect was found (35).

Benoxaprofen (Oraflex)

Benoxaprofen (Oraflex), a non-steroidal, anti-inflammatory medication, was used for rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and psoriasis (36). Eli Lilly & Co. voluntarily withdrew the drug from the market worldwide on August 4 1982. The company's decision came about once the United Kingdom suspended sales of the drug due to suspicion that it was associated with the death of 61 individuals (37) .The clinical trials had primarily taken place in the U.K. and the drug had been available for two years there (38). In the U.S., it had only been marketed for approximately three months before withdrawal. The pharmaceutical company had not reported fatalities when the FDA approved

the drug, however, soon after the U.K. reported 12 deaths (37). The patients had died from kidney and liver failure and all were elderly individuals taking the highest dose of 600 mg. Approximately two months before withdrawal, the FDA recommended 300 mg or a maximum of 450 mg as the starting dose for elderly individuals (37). At the time of withdrawal, there were possibly 11 deaths in the U.S. associated with the drug, but they had not presented with liver and kidney failure, as the cases in the U.K. (37). The British registry of adverse effects reported an additional 33 fatalities, 17 of which were caused by gastrointestinal hemorrhage or perforated ulcers (37).

Its long half-life of 25–32 hrs made a once daily dose convenient, however, in elderly patients the half-life could be extended to a dangerous 148 hours. The drug was metabolized by the liver and excreted in urine and feces. Its cutaneous adverse reactions included the following: photosensitivity in fair skinned individuals, oncholysis, cysts and milia, hypertrichosis, and Stevens–Johnson Syndrome (36). There was evidence that the drug was efficacious for prurigo nodularis and psoriasis (36). Psoriatic lesions have abnormally high levels of arachidonic acid (AA), and this compound is metabolized by either the cyclooxygenase or lipoxygenase pathway. Benoxaprofen served as stronger inhibitor of the latter pathway, in addition to inhibiting the chemotaxis of monocytes to psoriatic lesions (39). In a double blind randomized control study conducted in 1982, 40 subjects with psoriasis vulgaris were treated with 600 mg of the drug or placebo for 8 weeks (39). Seventy five percent of the treatment group showed improvement compared with minimal change in the control group (39). Its severe adverse effects were not reported in the study sample.

In its January 1988 issue, the British Medical Journal published an article entitled "Settlement of the benoxaprofen case" (38). The settlement agreement consisted of 2,275,000 pounds for 1200 plaintiffs. In the U.S, the scandal reached the press and on September 5, 1985 the New York Times reported that the U.S. Justice Department questioned Eli Lilly's actions or lack thereof (40). There was a high likelihood that the company was informed of the adverse effects of the drug and failed to report this to the FDA before its approval in the U.S. Unfortunately, many individuals were severely affected and fatalities occurred. It is important to keep in mind the interests and motivations of pharmaceutical companies for the sake of the individuals who will be consuming these drugs.

Chlorhexidine gluconate topical tincture 0.5% (Hibitane)

Chlorhexidine gluconate topical tincture 0.5% was indicated for use as a patient preoperative skin preparation, aiding in reducing skin infections. It was used on the skin prior to surgery or injections and contained 70% isopropyl alcohol (41).

The FDA approved it on December 18, 1978 and by early 1984, its sponsors, Steuart Pharmaceuticals and ICI Americas, now known as Zeneca Pharmaceuticals, voluntarily removed the product (42). A report published in 1985 discusses the thermal burns seven patients suffered from after the product was applied to their skin in preparation for electrocautery (41). According to the report, the skin must be given sufficient time to dry after prepping and before the procedure, in order to avoid burns. After withdrawal the FDA listed the product under the "Discontinued Drug Product List" in the Orange Book (41).

TABLE 65.4
Summary of Adverse Events in 95 Hemolytic Patients

Adverse Event	Number of Patients Affected	Percentage (%)
New onset renal dysfunction	54	57
Renal dysfunction requiring dialysis	34	63
Coagulopathy	33	35
Liver dysfunction	48	51
Central nervous system complication	4	4
Deaths	2	2

On May 24, 1996, an individual requested that the FDA consider the reasons why chlorhexidine gluconate topical tincture 0.5% was withdrawn, specifically whether it was due to the safety or effectiveness of the product (42). After review, the FDA established it was withdrawn for safety reasons since it caused chemical and thermal burns. The FDA removed chlorhexidine gluconate topical tincture 0.5% from the list of approved drug products and no longer accepted abbreviated new drug applications (ANDA's) (42).

Temafloxacin (Omniflox)

Temafloxacin, a broad spectrum fluoroquinolone antibiotic, was used to treat infections, including those involving the skin, prostate, lower respiratory tract, and urinary tract. The FDA approved it on January 5, 1992. Approximately four months later in June 1992, Abbott Laboratories voluntarily withdrew temafloxacin worldwide (43) because of reports of liver and renal failure, hemolytic anemia, and three deaths. These severe adverse events had not been recognized during clinical trials and no history of such severe adverse events existed with other fluoroquinolone antibiotics (43).

As early as April 1992, many young women without histories of underlying medical conditions, taking temafloxacin for urinary tract infections, developed hemolytic anemia (44,45). Subsequently, 95 cases were reported by June of the same year and this was termed the "temafloxacin syndrome" (46). In some cases, this syndrome was experienced after the first dose of treatment, especially in those with past quinolone use. Flank pain, chills, jaundice, dark colored urine, and a decrease in hemoglobin, were the signs and symptoms 6.4 days after beginning treatment. The mean drop in hemoglobin was 4.2 g/dL. The temafloxacin syndrome included acute renal failure in 2/3 of patients (some requiring dialysis), hepatobillary changes in 1/2 of patients, and coagulopathy in 1/3 of patients (Table 65.4) (45).

Allergic reactions causing respiratory distress were also documented along with low blood sugar in the elderly with renal problems. Two deaths were also reported (46). Health care professionals were critical in reporting these adverse events associated with temafloxacin.

Approximately, 189,000 individuals received a prescription for the drug (46). Of the patients with adverse reactions, 50% were using the drug for a respiratory infection, 28% for a genitourinary tract infection, and 7% for skin infections (46). Affected patients ranged from all ages. It is believed that temafloxacin caused

immune mediated hemolytic anemia, secondary to immune complex formation (46), however the mechanism by which a significant number of patients experienced the temafloxacin syndrome is not completely understood. The syndrome was presumably not identified during the premarketing studies, nor has an efficient animal model for this entity been identified. However, multiple renal effects and occult blood in urine were seen nonclinically in animal studies.

Astemizole 10 mg tablets (Hismanal)

Astemizole was withdrawn from the U.S. market by its manufacturer, Janssen Pharmaceutica, on July 18, 1999. Soon after the FDA stated that astemizole was removed for safety reasons. At this time, the FDA decided not to consider any ANDA's that included the drug product (47).

Janssen Pharmaceutica, received approval for this second generation antihistamine drug in 1988. Astemizole was indicated for seasonal allergic rhinitis and chronic idiopathic urticaria (47). However, as a histamine receptor blocker it also had severe adverse effects due to drug-drug interactions that could potentially cause torsades de pointes, (48) a ventricular tachycardia. This heart arrhythmia is characterized by prolongation of the QT interval and can be fatal.

The use of many drugs including clarithromycin, erythromycin, posaconazole, quinine, and ritonavir were contraindicated when using astemizole (49). These drugs could inhibit the metabolism of astemizole by a cytochrome P450 enzyme and by doing so prolong its half-life and cause fatal arrhythmias. Astemizole is a substrate of the enzyme CYP3A4, (50) and inhibition of this enzyme produces a dangerous increase in plasma levels of the drug. For example, a case was documented in which a patient receiving quinine sulfate for leg cramps and astemizole 10 mg/day, suffered from torsades de pointes due to the drug-drug interactions (50).

Interestingly, astemizole is found in Mexico under the brand name Antagon (51). In the U.S., Antagon is used for fertility and uses ganirelix as the active ingredient. However, in Mexico, Antagon is indicated for allergies and uses astemizole as the active ingredient. The FDA found that 17 other foreign drugs are marketed under the same brand name as an FDA approved drug in the U.S., but have a different active ingredient (51). This leaves room for confusion for individuals using these drugs. Currently, there is research delineating models for detecting proclivity for drugs producing torsades de pointes (52).

Terfenadine (Seldane)

Terfenadine, an antihistamine, used for rhino conjunctivitis and allergic skin conditions was once amongst the top 20 drugs most prescribed in the U.S. (53). The FDA approved it in 1985, and after over 10 years of marketing, was removed in 1997. At withdrawal, the drug had 24 million patient years experience (54). The key players in terfenadine's withdrawal involved the drug manufacturers, the FDA, and a patient case report. Marion Merrell Dow was assessing terfenadine for an OTC switch (54). It is more difficult to keep track of adverse side effects for OTC than prescription drugs, (54) but because there were no reports of significant adverse effects and it was presumed to be a safe drug, the additional steps were being taken to make the switch. However, upon further investigation, drug-drug interactions were evident.

Terfenadine is metabolized by CYP3A4 into fexofenadine and one other metabolite in the liver. If terfenadine is not metabolized, it stays in the plasma much longer, and the individual is at risk because at high levels, it blocks cardiac potassium channels (55). When potassium channels are blocked, repolarization is prevented, resulting in prolonged QT intervals and the patient is at risk for torsades de pointes. This condition is a potentially fatal ventricular arrhythmia, which is usually drug induced. Torsades de pointes was reported when administered with ketoconazole, an antifungal, or erythromycin, an antibiotic (56). In the absence of a metabolic inhibitor, for a patient taking terfenadine BID, the QT interval was normally prolonged by 6–8 msec during the dosing interval, and by 18 msec 1 h after the dose (57). Both COMPASS and HCHP studies with 180,000 and 20,000 subjects, respectively, did not find an increased risk of mortality with terfenadine use (57). However, when a metabolic inhibitor is present, the QT interval changes by 82 msec, and this significant alteration can be fatal (57).

The drug interaction between terfenadine and ketoconazole was first reported at Bethesda Naval Hospital in 1989 (58). A 39-year old woman taking terfenadine, cefaclor, ketoconazole, and medroxyprogesterone presented with a two-day history of dizziness and syncope. She had self-medicated with ketoconazole 200 mg bid for vaginal candidiasis and was now experiencing torsades de pointes with a QTc of 655 msec (55). This was the first documented case of torsades de points in a patient taking terfenadine that was not due to a drug overdose. The FDA then investigated terfenadine and its drug-drug interactions by providing funding to Georgetown University to work alongside the FDA and the Uniformed Services University in conducting clinical studies (58). The team discovered that terfenadine, astemizole, mebefradil, and cisapride could all lead to a ventricular tachycardia when taken with other drugs. At the time, physicians also began to report torsades de pointes in patients taking terfenadine with ketoconazole or erythromycin.

The FDA proposed withdrawal of terfenadine because although the risks had been repeatedly communicated to physicians, the drug was still being prescribed concomitantly with other drugs that inhibited its metabolism (59). This was a preventable risk that unfortunately could not be put into practice 100% of the time. In January of 1997, terfenadine's manufacturers removed the drug following the approval of fexofenadine (Allegra), a safer alternative (60).

Post marketing surveillance is essential in managing the risks and benefits of a drug. Clinical trials are unable to document every possible side effect, considering their limited sample size ranging from hundreds to thousands of subjects. A rare adverse event can be missed during trials that has the possibility of affecting a significant number of individuals in the general population. There are many factors to consider in evaluating the risks and benefits of a drug. Terfenadine's history highlights this difficulty. The frequency, severity, and reversibility of an adverse effect must be weighted against drug efficacy (61). In addition, individual variability in drug metabolism and individual practices can also play an integral part. Individuals in the U.S. take many prescription drugs with half of the U.S. population taking at least one prescription drug a week in a given week and 7% taking at least five (62). The number of medications increases drastically when OTC, vitamins/minerals, and herbal supplements are included (62). Health care professionals also have the important responsibility of reporting side effects their patients experience with drug use.

With current models for analyzing proclivity to torsades de pointes, this risk may be identifiable for new drugs. Clearly, this

experience points to the opportunities for improving post-marketing surveillance. We now have electronic data, improved adverse event assessment, and risk evaluation and mitigation strategy (REMS) for post-marketing.

Etretinate (Tegison)

Etretinate received FDA approval on September 30, 1986 and was voluntarily withdrawn from the US market on December 20, 2002 by its manufacturers, Hoffmann-La Roche (63). The drug was used for the treatment of severe, recalcitrant psoriasis.

Etretinate was the second oral retinoid approved by the FDA and like all compounds in this class, posed the risk of teratogenicity (64). This risk was well supported in animal studies and in humans with the use of isotretinoin, the first oral retinoid. For this reason, it was contraindicated in pregnancy as category X and had a boxed warning in its labeling. At the time of approval, the Isotretinoin Pregnancy Prevention Program had not been implemented (64). Cases of both neurologic and skeletal deformities in fetuses were reported.

Patients on etretinate were required to use contraceptives a month before and for a significant time after treatment. Etretinate's lowest teratogenic dose in humans is 0.2 mg/kg/day (65). As much as 2.9 years after treatment had been discontinued, blood levels in some individuals ranged from 0.5 to 12 ng/mL (66). Pregnancy had to be avoided at least three years after terminating treatment (67). The drug was so potent that individuals who had taken etretinate were permanently excluded from donating blood or plasma, due to the possibility of transfusing blood products to someone who was pregnant or planned to become pregnant (66).

Although retinoids are necessary for normal epithelial proliferation, differentiation, and embryo fetal development, oral retinoids can cause serious adverse effects. Oral retinoids have many systemic effects affecting the liver, thyroid, eyes, and lipid metabolism (68). Mucocutaneous, cutaneous, and musculoskeletal effects are also seen (68). These clinical side effects are due to the receptor subtypes to which they bind and activate. The retinoids act as teratogens by preventing neural crest cells from carrying out their normal activity and migration during development. As a consequence, craniofacial, thymic, heart, and central nervous system abnormalities result.

Hoffmann-La Roche discontinued marketing of the drug in 1999 and requested the FDA to withdraw approval (63). On December 30, 2002, the FDA responded with an acknowledgment letter stating that because of its greater teratogen risk, etretinate was removed from the market (63).

Acetretin was approved by the FDA in 1996, and is an active metabolite of etretinate. It is also indicated for the treatment of severe, recalcitrant psoriasis. While etretinate is stored in adipose tissue and has a half-life of 120 days, acetretin has the advantage of having a much shorter half-life of only two days and not accumulating in adipose tissue (66). This allows for a shorter period of time for the risk of birth defects in women who have discontinued the drug.

More detailed information on drug metabolism, drug-drug, and drug-food interactions may help identify these risks in the future.

Efalizumab (Raptiva)

In October 2003, the FDA approved Genentech's efalizumab for use in the treatment of moderate to severe plaque psoriasis in adults. A once-weekly injection suppressed T-cells of the immune system and prevented these cells from attacking the skin and producing inflammation. At the same time that it helped decrease the amount of scaly patches, it also suppressed the entire immune system, leaving the patient vulnerable to infections (69).

TABLE 65.5
Adverse Events in Patients Taking Efalizumab vs Placebo

Adverse Event	Placebo (n = 715) (%)	Efalizumab 1 mg/kg/wk (n = 1213) (%)
Headache	159 (22)	391 (32)
Infection	188 (26)	350 (29)
Chills	32 (4)	154 (13)
Nausea	51 (7)	128 (11)
Pain	38 (5)	122 (10)
Myalgia	35 (5)	102 (8)
Flu syndrome	29 (4)	83 (7)
Fever	24 (3)	80 (7)
Back pain	14 (2)	50 (4)
Acne	4 (1)	45 (4)

Genentech announced voluntary withdrawal in April 2009, approximately 4.5 years after its initial FDA approval and by June 8, 2009 the drug was no longer available in the U.S. (70). Drug sales in Europe and Canada were suspended by the European Medicines Agency (EMEA) and Serono Canada, Inc., respectively, in February 2009 (71). Efalizumab was withdrawn because of the risk of progressive multifocal leukoencephalophathy (PML) (70), a serious and often fatal neurologic disease caused by a virus that affects the central nervous system. Symptoms include weakness, vision and speaking difficulties, and loss of coordination, leading to severe disability. It is a rare disease that usually occurs in people whose immune system has been significantly weakened.

A series of FDA actions and petitions eventually led to the drug's withdrawal. On October 16, 2008, the FDA called for a boxed warning on efalizumab labeling to warn of the risk of life threatening infections, such as PML (72). At this time, the FDA also required Genentech to provide a REMS for patients (73). On February 19, 2009 the FDA issued a public health advisory, announcing the risk of PML in patients taking efalizumab (72). At this point, the FDA had received three reports of confirmed cases and one possible case of PML in patients taking the drug (74). Three of the four resulted in fatalities. Affected individuals were between 47–73 years old, had used efalizumab for over three years, and were not on any other treatment or drug that could cause weakening of their immune system (74). Genentech developed a medication guide for the drug and the FDA approved it in March 2009, providing additional information on the risk of PML. Finally, on April 8, 2009, Genentech voluntary withdrew the drug from the U.S. market.

Although four PML cases were reported to the FDA in the post-marketing setting, none of the 2,764 patients who participated in their clinical trials developed PML at that time. However, a longer period of time on the drug during clinical trials may have been required for the development of PML since of the 2762 patients, 2400 were treated for three months, 904 for six months, and only 218 for more than a year (75). Clinical trial patients had a median age of 44 years, 67% were male, and 89% were Caucasian. The reported adverse events in placebo controlled clinical trials reported at a $\geq 2\%$ higher rate in the 1 mg/kg/wk efalizumab treatment compared with the placebo group (Table 65.5) (75).

TABLE 65.6
Summary of Withdrawn Drugs, Indications, and Side Effects

Drug	FDA Approval Year	Withdrawal Year	Approved Use	Reason for Withdrawal
Bithionol	_____	1967	Antibacterial agent in lotions, shampoos, creams, etc.	Photodermatitis
Dibromsalan	_____	1975	Antimicrobial agents found in soaps and cosmetic products	Photodermatitis
Metabromsalan	_____	1975	Antimicrobial agents found in soaps and cosmetic products	Photodermatitis
Tribomosalan	_____	1975	Antimicrobial agents found in soaps and cosmetic products	Photodermatitis
3,3,4,5-Tetrachlorosalicylanilide	_____	1975	Antimicrobial agents found in soaps and cosmetic products	Photodermatitis
Chloroform	_____	1976	Found in cosmetics, topical liniments, and toothpaste	Carcinogenic in animals
Azaribine (Triazure tablets)	1975	1977	Psoriasis treatment	Thromboembolic events
Zirconium aerosol	_____	1977	Antiperspirants	Human skin granulomas
Potassium arsenite (Fowler's Solution)	_____	1980	Lepra, prurigo, and psoriasis	Carcinogen
Chlorohexidine gluconate topical tincture (Hibitane)	1978	1984	Preoperative skin preparation	Thermal burns
Temafloxacin (Omniflox)	1992	1992	Antibiotic (used to treat skin infections)	Kidney and failure, Hemolytic anemia
Terfenadine (Seldane)	1985	1997	Antihistamine	Torsades de pointes
Astemizole (Hismanal)	1988	1999	Antihistamine	Torsades de pointes
Etretinate (Tegison)	1986	2002	Psoriasis	Birth defects
Efalizumab (Raptiva)	2003	2009	Psoriasis	Progressive multifocal leukoencephalopathy (PML)

In addition, eight cases of thrombocytopenia and 19 cases of serious psoriasis events were reported in the treatment group (75). Two cases of hemolytic anemia were observed during these clinical trials and two more cases were reported post marketing (76).

Current clinical trials are insufficient to identify uncommon intolerances. They require larger sample sizes and a longer duration. However, this necessitates increases in expenditure and in some cases a delay in treatment in patients who may need a new drug. In the case of efalizumab, many side effects were seen more often in the treatment than in the placebo group. However, there is no data available on whether these differences are statistically significant. Having the benefits outweigh the side effects of a drug is a difficult challenge, but one that is necessary. Patients take these medications to cure or control their illnesses, not to cause them extreme disability and even death. Many times a physician prescribes a medication for the good of the patient, without knowing the risks of taking a drug. The extra step would be to explain to patients that clinical trials of the drugs do not necessarily establish 100% of the possible side effects. Although fear may be instilled in the patient by doing so, this would prevent the unpleasant side effects from being such a surprise when taking certain drugs. Prospective registration of patients receiving biologics in Sweden and the United Kingdom should enhance our knowledge of risk (77). In the U.S., REMS have been required from manufacturers since 2007 for drug approval. In this way, the FDA evaluates risks versus benefits of a drug, and approves only those that meet the safety standard.

DISCUSSION

After a review of withdrawn drugs, 16 had a dermatologic use. Some were antimicrobial components in soaps or used as skin antibiotics, while others were indicated for psoriasis and allergies (Table 65.6). All were withdrawn for safety reasons. With the exception of chloroform, indisputable evidence exists in which individuals exposed to these drugs experienced adverse side effects. The question remains whether the reported side effects could have been predicted before approval by the FDA in order to prevent marketing in the U.S.

In the case of bithionol and halogenated salicylanilides, prior knowledge of their phototoxic effect would have most likely impeded their marketing. Testing for photoallergic contact dermatitis was not routine at the time. In today's scientific community, testing for phototoxicity of a new drug/substance that is suspected of having a phototoxic effect is straightforward and essential in ensuring safety to the public.

Both astemizole and terfenadine posed the risk of drug-drug interactions leading to torsades de pointes. Inhibition of the enzyme that metabolizes them increased their half-life and severe consequences resulted. It is difficult to predict such interactions during clinical trials, especially when individuals may be taking a variety of drugs, including prescribed, OTC, and herbal medications. However, drug-drug interactions are now checked both in vitro and in vivo in humans. Not only is informing physicians of such drug-drug interactions a challenge, but also having physicians themselves inform their patients. In the case of these two drugs, possibly having a larger sample size during their clinical trials in which all medications their participants were using were kept track of would have been helpful.

Patients taking efalizumab and temafloxacin suffered debilitating and in some cases fatal side effects. By suppressing the entire immune system, efalizumab put patients at risk for PML. Indeed, it was expected that there would be infections or cancer, secondary to immunosuppression. Temafloxacin on the other hand, could cause liver and renal failure and hemolytic anemia. In both cases, side effects were not reported during clinical trials, but renal and hemolytic problems were predicted nonclinically. It is possible that a larger sample size and/or longer duration of the clinical studies was required to witness these side effects. As expensive as clinical trials may be, falling short of the most thorough review of the effects of a drug can have fatal consequences and this needs to be avoided at all costs.

Unlike all other drugs described here, the severe side effects of azarabine were reported during clinical trials, yet it was still approved. This drug was indicated for psoriasis patients and this group of individuals was thought to be at higher risk for thromboembolic events. Thus, when vascular problems were reported during clinical trials, they were not attributed to the drug. The learning point from this example is to never overlook a side effect. Patients believe their medications are safe to take and in most cases do not expect use of these drugs to cause severe side effects.

The case of benoxaprofen was unique in that the drug had been marketed in the U.K. for over three years and at the time of FDA approval, fatalities associated with kidney and liver failure had not been reported. Perhaps a more thorough FDA investigation independent of the pharmaceutical company would have highlighted adverse effects earlier and not only prevented its approval in the U.S., but also reduced the number of fatalities in the U.K.

Use of both chloroform and potassium arsenite were linked to cancer. Chloroform was withdrawn because it was a reasonably anticipated human carcinogen since it caused cancer in animal studies. (Of note, chloroform is currently allowed as a solvent impurity.) In humans, only associations were made. However, in the case of potassium arsenite, a direct causal relationship was found and it was removed from the market.

Chlorhexidine gluconate topical tincture 0.5% was used in skin preparations mainly prior to surgery. It was removed after reports of thermal burns. Its 1978 approval assumes this side effect was not reported during clinical trials. Once again, whether more extensive clinical trials could have prevented individuals from being affected is unknown. For zirconium aerosol products used as antiperspirants, the history is similar. They were on the market in the 1950s and caused granuloma formation in human skin. These OTC products may have not undergone extensive safety testing prior to being available to consumers.

Lastly, in the case of etretinate, its teratogenic effects were known and patients were extensively warned. The drug was removed from the market once a safer alternative was available.

Since its existence, the FDA has continuously revised its protocols and standards for drug approval. Stricter measures along with increased knowledge about side effects have minimized danger to the public. However, as the drugs discussed in this report illustrate, it is a challenge to ensure the efficacy of a drug while ensuring safety.

These 16 withdrawn drugs, all had a dermatologic purpose. The skin is a complicated organ to treat as highlighted by the history of each drug discussed in this report. Cancer, PML, liver and renal failure, granulomas, teratogenicity, phototoxicity, and drug-drug interactions are all serious side effects that need to be avoided for the sake of the patient's well being. Patients' faith in physicians partly relies on receiving the correct medications that will return them to good health and not harm them. Doing as much as necessary to ensure safety must be a priority, as current clinical trials for new drugs exemplify. Taken together, increasing dermatoxic knowledge and experience should decrease the number of future withdrawals (78).

ACKNOWLEDGMENTS

Special thanks to Dr. Abigail Jacobs for her contribution to this manuscript.

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About the authors

Klaus-Peter Wilhelm, M.D., is extraordinary professor of dermatology at the University of Lübeck, Germany, and president and medical director of proDERM Institute for Applied Dermatological Research Schenefeld/Hamburg, Germany. He is President of the International Society for Biophysics and Imaging of the Skin, and a member of several scientific associations and societies. He has published over 100 manuscripts and reviews and coauthored three books in the Bioengineering of the Skin series. Dr. Wilhelm received an M.D. degree from the Medical University of Lübeck, Germany.

Hongbo Zhai, M.D., is a senior research fellow at the Department of Dermatology in the University of California at San Francisco, USA. He has over two decades of experience in the prevention of contact dermatitis and the development of skin disease-related products. He has contributed to the development of innovative skin-related products in collaboration with many global pharmaceutical and cosmetic companies. He has published more than 100 scientific articles in his research areas. Dr. Zhai is also the 2003 winner of the international "Niels Hjorth Prize."

Howard I. Maibach, M.D., is professor of dermatology in the School of Medicine at the University of California, San Francisco, USA. He has several decades of research experience in skin diseases and the development of skin-related products. He has published more than 2000 papers and over 80 textbooks. He is a consultant to government agencies, universities, and industry.

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ISBN 978-184184855-6
9 781841 848556