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Safety Terminology

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INTRODUCTION

One of the skin's primary physiological functions is to act as the body's first line of defense against exogenous agents. However, the skin should not be viewed as a flawless physicochemical barrier. Many low–molecular weight compounds are capable of penetrating this barrier. When toxic agents (such as irritants or allergens in cosmetic products) permeate it, the resulting adverse effects may cause considerable discomfort to the consumer. Even minor disturbances of the skin surface can produce discomfort, especially in the facial area which has an extensive network of sensory nerves. Moreover, because most cosmetics are applied to the highly permeable facial skin, the majority of reported cosmetic reactions occur in the face. Therefore, safety with regard to cosmetic products is a vital issue.

This chapter provides a brief summary of the safety terminology pertaining to cosmetic reactions, as well as an overture to the succeeding chapters. The reader is directed toward some in-depth reviews of each topic in the bibliography.

CONTACT DERMATITIS

This is a nonspecific term used to describe any inflammatory skin disease resulting from contact with an irritant or allergenic substance. Whatever the causative agent, the clinical features are similar: itching, redness, and skin lesions. It is also often used (inaccurately) as a synonym for allergic contact dermatitis (ACD).

IRRITANT CONTACT DERMATITIS (IRRITATION)

Irritant contact dermatitis (ICD) is a term given to a complex group of localized inflammatory reactions that follow nonimmunological damage to the skin. The inflammation may be the result of an acute toxic (usually chemical) insult to the skin, or of repeated and cumulative damage from weaker irritants (chemical or physical). There is no definite laboratory test for ICD—diagnosis is by clinical morphology, of course, and appropriate negative patch-test results.

Irritant

An irritant is any agent, physical or chemical, that is capable of producing cell damage if applied for sufficient time and in sufficient concentration. Irritants can produce a reaction in anyone, although individual susceptibility varies. The clinical reaction produced by irritants varies considerably.

Acute Irritant Contact Dermatitis

Acute ICD is the result of a single overwhelming exposure to a strong irritant or a series of brief physical or chemical contacts, leading to acute inflammation of the skin. The resultant clinical appearance is that of erythema, edema, pain, and sometimes vesiculation at the site of contact, usually associated with burning or stinging sensations.

Irritant Reaction

An irritant reaction is a transient noneczematous dermatitis characterized by erythema, chapping, or dryness, and resulting from exposure to less potent irritants. Repeated irritant reactions may lead to contact dermatitis.

Cumulative Irritant Contact Dermatitis

Cumulative irritant contact dermatitis or chronic ICD develops as a result of a series of repeated and damaging insults to the skin. The insults may be chemical or physical.

Delayed Acute Irritant Contact Dermatitis

Some chemicals produce acute irritation in a delayed manner so that the signs and symptoms of acute irritant dermatitis appear 12 to 24 hours or more after the original insult.

Subjective (Sensory) Irritation

This refers to sensations of burning, stinging, and itching that are experienced by certain susceptible individuals after contact with certain chemicals, although no visible inflammatory pathology can be seen. Examples of sensory irritants in cosmetics are lactic acid, salicylic acid, propylene glycol, and some benzoyl peroxide preparations.

ALLERGIC CONTACT DERMATITIS

ACD occurs when a substance comes into contact with skin that has undergone an acquired specific alteration in its reactivity as a result of prior exposure of the skin to the substance eliciting the dermatitis. The skin response of ACD is delayed, immunologically mediated (Type IV), and consists of varying degrees of erythema, edema, papules, and papulovesicles. Patch testing is the gold standard; it is imperative for proving ACD, determining the actual allergen, predictive testing, i.e., determining ‘‘safe’’ materials for the consumer, and exclusion of other diagnoses.

Allergen

Allergens are low–molecular-weight (<500–1000 Da) molecules capable of penetrating the skin and binding to skin proteins to form a number of different antigens that may

stimulate an allergic response in an individual. Common allergens in cosmetic products are fragrances (e.g., cinnamic aldehyde) and preservatives (e.g., formaldehyde and formaldehyde donors).

PHOTOIRRITANT CONTACT DERMATITIS (PHOTOIRRITATION/PHOTOTOXICITY)

Photoirritant contact dermatitis (PICD) is a chemically induced nonimmunological skin irritation requiring light. This reaction will occur in all individuals exposed to the chemical–light combination. The clinical picture is that of erythema, edema, or vesiculation in sun-exposed areas, resembling an exaggerated sunburn. This may be followed by hyperpigmentation, or if the exposure is repeated, scaling and lichenification may occur. Bergapten, a component of bergamot oil, which used to be a popular ingredient in perfume, is a potent photoirritant that causes berloque dermatitis.

PHOTOALLERGIC CONTACT DERMATITIS

Photoallergic contact dermatitis (PACD) is an immunological response to a substance that requires the presence of light. The substance in the skin absorbs photons and is converted to a stable or unstable photoproduct, which binds to skin proteins to form an antigen, which then elicits a delayed hypersensitivity response. Examples of photoallergens present in cosmetics are musk ambrette and 6-methylcoumarin, which are present in fragrances. Photopatch testing is the diagnostic procedure for photoallergy.

CONTACT URTICARIA SYNDROME

Contact urticaria syndrome (CUS) represents a heterogeneous group of inflammatory reactions that appear, usually within a few minutes to an hour, after contact with the eliciting substance. Clinically, erythematous wheal-and-flare reactions are seen, and sensations of burning, stinging, or itching are experienced. These are transient, usually disappearing within a few hours. In its more severe forms, generalized urticaria or extracutaneous manifestations, such as asthma, nausea, abdominal cramps, and even anaphylactic shock, may occur. Diagnosis may be achieved by a variety of skin tests—the open test is the simplest of these and is the ‘‘first-line’’ test.

CUS may be divided into two categories on the basis of pathophysiological mechanisms: nonimmunological and immunological. There are also urticariogens that act by an uncertain mechanism.

Nonimmunological Contact Urticaria

Nonimmunological contact urticaria (NICU), which occurs without prior sensitization, is the most common class of CUS. The reaction usually remains localized. Examples of cosmetic substances known to produce NICU are preservatives (e.g., benzoic acid and sorbic acid) and fragrances (e.g., cinnamic aldehyde).

Immunological Contact Urticaria

Immunological contact urticaria (ICU) are immediate (Type I) allergic reactions in people who have previously been sensitized to the causative agent. ICU is IgE mediated and is more common in atopic individuals. Food substances are common causes of ICU.

ACNEGENICITY

This refers to the capacity of some agents to cause acne or aggravate existing acne lesions. This term may be subdivided to include comedogenicity and pustulogenicity.

Comedogenicity

This is the capability of an agent to cause hyperkeratinous impactions in the sebaceous follicle, or the formation of microcomedones, usually in a relatively short period of time.

Pustulogenicity

This refers to the capability of an agent to cause inflammatory papules and pustules, usually in a relatively short period of time.

SENSITIVE SKIN

This term is a neologism for consumers' feelings about their intolerance to a variety of topical agents, be it topical medicaments or cosmetics and toiletries. Individuals present with very similar complaints, such as burning, stinging or itching sensations, on contact with certain cosmetic products that most people do not seem to react to, sometimes accompanied by slight erythema or edema. They frequently complain of a ''tight feeling'' in their skin, secondary to associated dry skin. Sensitive skin describes the phenotype noted by the consumer; mechanisms include sensory irritation, suberythematous irritation, acute and cumulative irritation, contact urticaria, allergic contact dermatitis, as well as photoallergic and phototoxic contact dermatitis. Sensory irritation and suberythematous irritation are believed to be far more common than the remaining mechanisms.

Cosmetic Intolerance Syndrome

The term cosmetic intolerance syndrome (CIS) is applied to the multifactorial syndrome in which certain susceptible individuals are intolerant of a wide range of cosmetic products. CIS is thought to be caused by one or more underlying occult dermatological conditions, such as subjective irritation, objective irritation, allergic contact dermatitis, contact urticaria, or subtle manifestations of endogenous dermatological diseases, such as atopic eczema, psoriasis, and rosacea.

Status Cosmeticus

Status cosmeticus is a condition in which every cosmetic product applied to the face produces itching, burning or stinging, rendering the sufferer incapable of using any cosmetic product. The patient's history usually includes ''sensitivity'' to a wide range of products. This diagnosis is only declared after a full battery of tests have proved negative, and may be considered the extreme end of the spectrum of sensitive skin.

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Principles and Practice of Percutaneous Absorption

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INTRODUCTION

Percutaneous absorption is a complex biological process. The skin is a multilayered biomembrane that has certain absorption characteristics. If the skin were a simple membrane, absorption parameters could easily be measured, and these would be fairly constant provided there was no change in the chemistry of the membrane. However, skin is a dynamic tissue and as such its absorption parameters are susceptible to constant change. Many factors and skin conditions can rapidly change the absorption parameters. Additionally, skin is a living tissue and it will change through its own growth patterns, and this change will also be influenced by many factors. This chapter reviews some of the principles and technologies of percutaneous absorption for developers and users of cosmetics.

STEPS TO PERCUTANEOUS ABSORPTION

A cosmetic that comes in contact with human skin will be absorbed into and through the skin. The components of the cosmetic will respond to the chemical and physical laws of nature, which direct the absorption process. Examples of this are solubility, partition coefficients, and molecular weight. The skin presents a barrier, both physical structure and chemical composition. A cosmetic component will transverse from a lipophilic stratum corneum to a more progressively hydrophilic epidermis, dermis, and blood microcirculation.

Percutaneous absorption has been defined as a series of steps [1]. Table 1 lists our current knowledge of these steps. Step 1 is the vehicle containing the chemical(s) of interest. There is a partitioning of the chemical from the vehicle to the skin. This initiates a series of absorption and excretion kinetics that are influenced by a variety of factors, such as regional and individual variation. These factors moderate the absorption and excretion kinetics [2].

Once a chemical has been absorbed through the skin, it enters the systemic circulation of the body. Here, the pharmacokinetics of the chemical define body interactions. This is illustrated for [^{14}C]hydroquinone in vivo in man, where plasma radioactivity was measured ipsilaterally (next to the dose site) and contralaterally (in the opposite arm) after a topical dose. Thirty minutes after the dose, the hydroquinone has been absorbed through the skin and has reached a near-peak plasma concentration (Fig. 1) [3]. Figure 2 shows

TABLE 1 Steps to Percutaneous Absorption

Vehicle
Absorption kinetics
Skin site of application
Individual variation
Skin condition
Occlusion
Drug concentration and surface area
Multiple-dose application
Time
Excretion kinetics
Effective cellular and tissue distribution
Substantivity (nonpenetrating surface adsorption)
Wash and rub resistance/decontamination
Volatility
Binding
Anatomical pathways
Cutaneous metabolism
Quantitative structure activity relationships
Decontamination
Dose accountability
Models

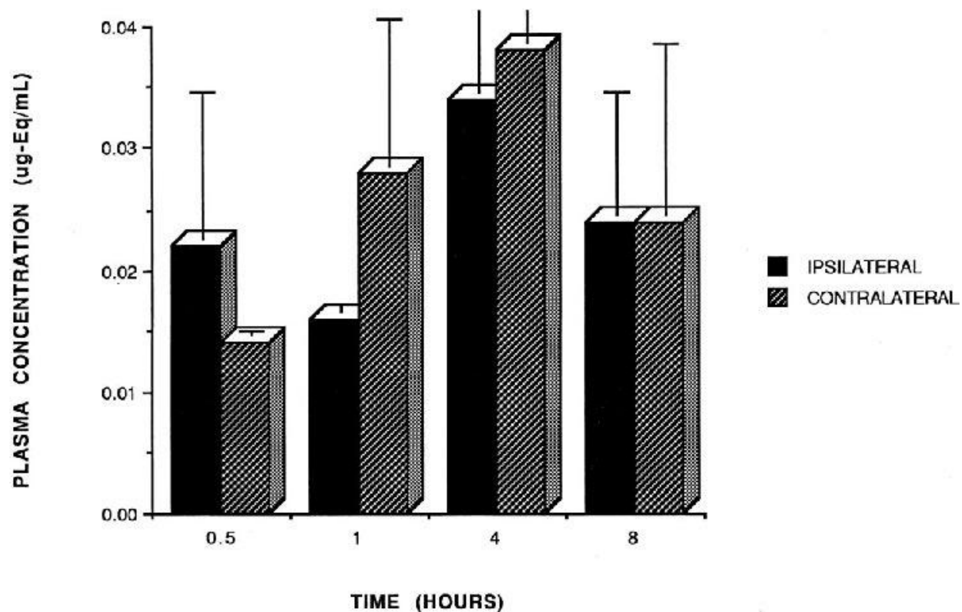

FIGURE 1 Plasma radioactivity is detected in human volunteers 30 minutes after [^{14}C]hydroquinone is applied to skin. Ipsilateral is blood taken near the site of dosing, and contralateral is from the other arm. Hydroquinone is rapidly absorbed into and through human skin.

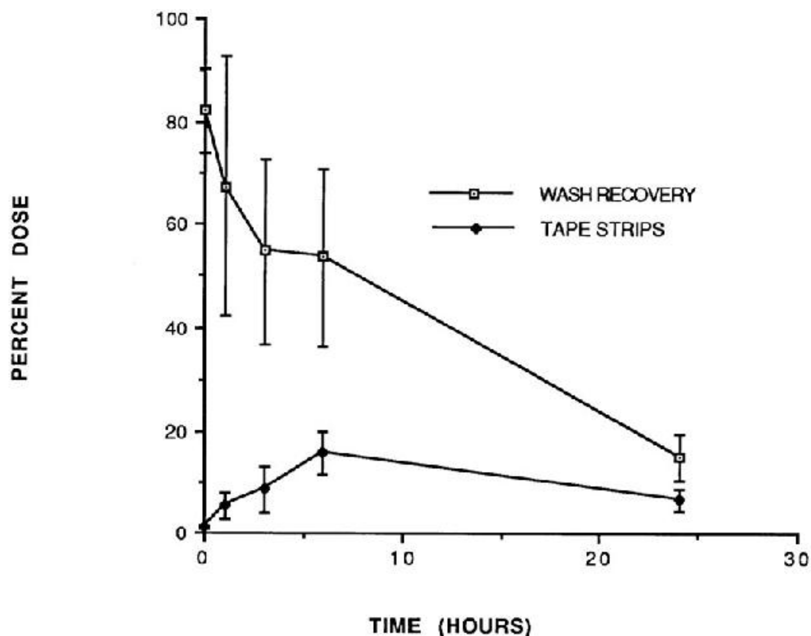

FIGURE 2 Hydroquinone is applied to human skin. Wash recovery with time decreases because hydroquinone is being absorbed into and through human skin. At the same time, tape strips of the skin surface show a rise in stratum-corneum content of hydroquinone. It is a dynamic process; hydroquinone disappears from the skin surface, appears and increases in the stratum corneum, and then appears in the blood.

hydroquinone disappearance from the surface of the skin (decreased wash recovery) and concurrent appearance in the stratum corneum (obtained from skin tape strips) [3]. As the cosmetic component transverses the skin, the chemical can be exposed to skin enzymes, which are capable of altering the chemical structure through metabolism [3].

METHODS FOR PERCUTANEOUS ABSORPTION

Ideally, information on the dermal absorption of a particular compound in humans is best obtained through studies performed on humans. However, because many compounds are potentially toxic, or it is not convenient to test them in humans, studies can be performed using other techniques. Percutaneous absorption has been measured by two major methods: (1) in vitro diffusion cell techniques, and (2) in vivo determinations, both of which generally use radiolabeled compounds. To ensure their applicability to the clinical situation, the relevance of studies using these techniques must constantly be challenged [4].

In vitro techniques involve placing a piece of human skin in a diffusion chamber containing a physiological receptor fluid. The compound under investigation is applied to one side of the skin. The compound is then assayed at regular intervals on the other side of the skin. The skin may be intact, dermatomed, or separated into epidermis and dermis; however, separating skin with heat will destroy skin viability. The advantages of

the in vitro techniques are that they are easy to use and results are obtained quickly. Their major disadvantage is the limited relevance of the conditions present in the in vitro system to those found in humans.

Percutaneous absorption in vivo is usually determined by the indirect method of measuring radioactivity in excreta after the topical application of a labeled compound. In human studies, the plasma level of a topically applied compound is usually extremely low—often below assay detection. For this reason, tracer methodology is used. After the topical application of the radiolabeled compound, the total amount of radioactivity excreted in urine or in urine plus feces is determined. The amount of radioactivity retained in the body or excreted by a route not assayed (CO_2) is corrected for by determining the amount of radioactivity excreted after parenteral administration. Absorption represents the amount of radioactivity excreted, expressed as percentage of the applied dose. Percutaneous absorption can also be assessed by the ratio of the areas under the concentration-versus-time curves after the topical and intravenous administration of a radiolabeled component. The metabolism of a compound by the skin as it is absorbed will not be detected by this method. A biological response, such as vasoconstriction after the topical application of steroids, has also been used to assess dermal absorption in vivo [4].

An emerging method is that of skin tape stripping. After washing, consecutive stratum corneum tape strips exhibit a profile, such as that for estradiol (Fig. 3) in human stratum corneum. The first few strips have higher estradiol content because they contain residual surface estradiol. Tape stripping can show a profile of a cosmetic within skin

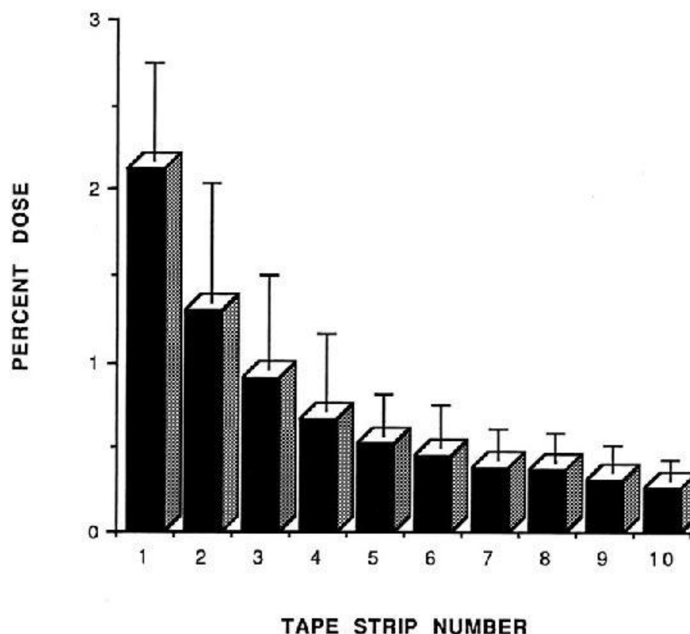

FIGURE 3 Estradiol is applied to human skin, then washed 24 hours after dosing. Tape strips (consecutive 1–10 in some areas) show a concentration pattern of estradiol through the stratum corneum.

over a time course. In addition, the chemical content of the tape strippings can be used to compare bioavailability of competing products. Proof can be obtained by using this technique to observe which products penetrate skin faster and deeper.

INDIVIDUAL AND REGIONAL VARIATION

In vivo and in vitro percutaneous absorption studies give data as mean absorption ± some standard deviation. Some of this variability is attributable to conduct of the study and is called *experimental error*. However, when viewing a set of absorption values it is quite clear that some people (as well as some rhesus monkeys) are low absorbers and some are high absorbers. This becomes evident with repeat studies. This is *individual variation*.

The first occupational disease in recorded history was scrotal cancer in chimney sweeps. The historical picture of a male worker holding a chimney brush and covered from head to toe with black soot is vivid. But why the scrotum? Percutaneous absorption in humans and animals varies depending on the area of the body on which the chemical resides. This is called *regional variation*. When a certain skin area is exposed, any effect of the chemical will be determined by how much is absorbed through the skin. Feldmann and Maibach [5–7] were the first to systemically explore the potential for regional variation in percutaneous absorption. The first absorption studies were performed on the ventral forearm because this site is convenient to use. However, skin exposure to chemicals exists over the entire body. The scrotum was the highest-absorbing skin site (scrotal cancer in chimney sweeps is the key). Skin absorption was lowest for the foot area, and highest around the head and face (Fig. 4). There are two major points. First, regional variation was confirmed with the different chemicals. Second, those skin areas that would be exposed to cosmetics—the head and face—were among the higher absorbing sites.

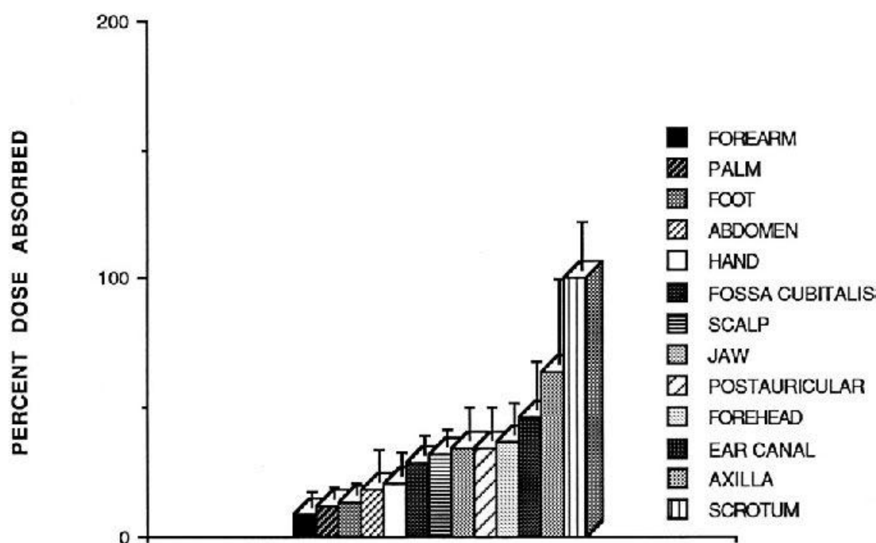

FIGURE 4 Percutaneous absorption of parathion from various parts of the body varies with region of the body.

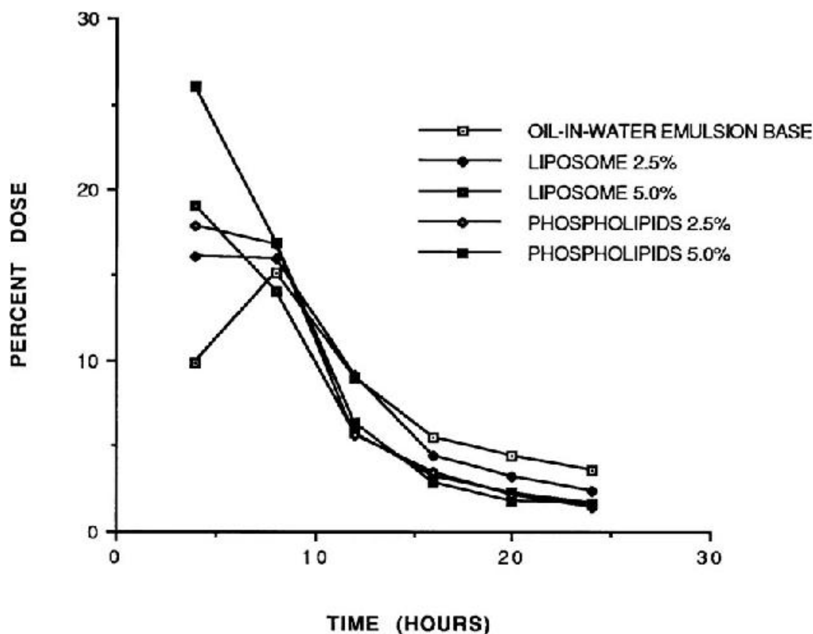

FIGURE 5 Lidocaine percutaneous absorption through human skin. Formulation determines the initial absorption.

VEHICLE INFLUENCE ON PERCUTANEOUS ABSORPTION

A cosmetic can be a single ingredient or a mixture of chemicals in a vehicle. The vehicle can have a great effect on skin absorption of the chemical(s). Lidocaine was applied to human skin in an in vitro absorption study. Figure 5 shows receptor fluid (circulating under the skin to collect absorbed lidocaine) accumulation with time. Initially the vehicle had a great influence on the partitioning of lidocaine into the skin. With time, the influence of the vehicle decreased and lidocaine absorption was constant for all vehicles. Interestingly, when the lidocaine content of epidermis and dermis was determined, there was more lidocaine retained by the oil-in-water (o/w) emulsion (Fig. 6). Vehicles can direct chemical distribution within skin and this can be validated with the proper experiment.

There is also an interesting vehicle effect for multiple dosing on skin. A multiple dose exceeds that predicted by absorption from single-dose administration (Fig. 7). The hypothesis is that the second and subsequent dosed vehicles ''reactivate/solubilize'' the initial chemical from skin binding and push the chemical further down into and through the skin [8].

SKIN CLEANSING AND DECONTAMINATION

Although decontamination of a chemical from the skin is commonly performed by washing with soap and water (because it is largely assumed that washing will remove the chemical), recent evidence suggests that the skin and the body are often unknowingly subjected to enhanced penetration and systemic absorption/toxicity because the decontamination procedure does not work or may actually enhance absorption [9].

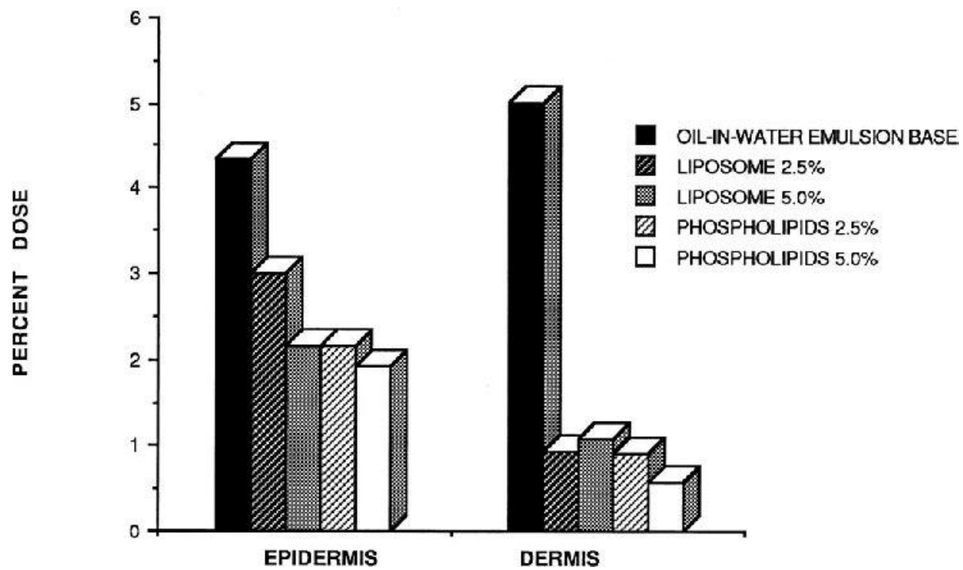

FIGURE 6 Distribution of lidocaine in human epidermis and dermis. Formulation determines the concentration within the skin component.

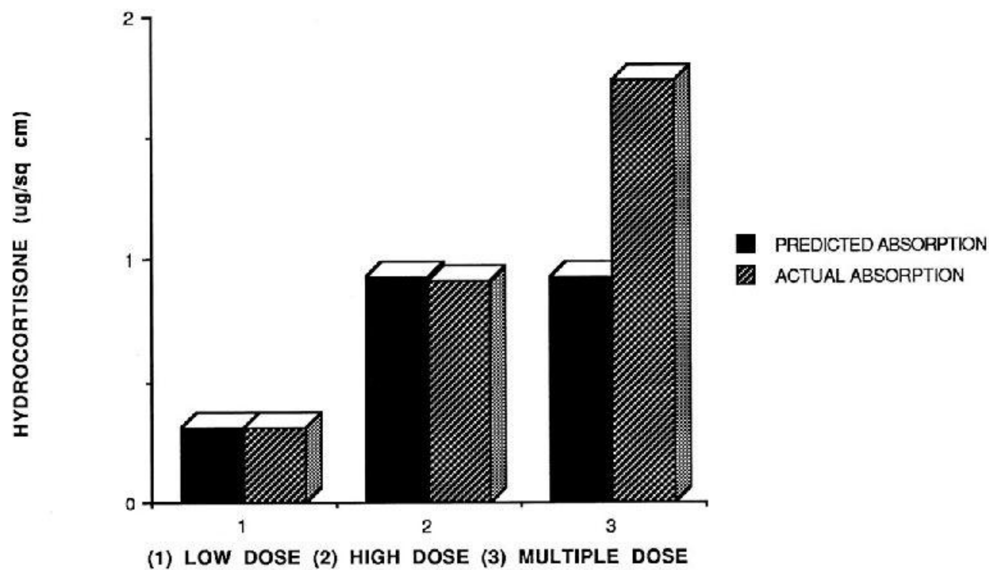

FIGURE 7 Hydrocortisone in cream base was dosed on human skin as a low dose (x) and a high dose (3x). When the low dose (x) was dosed three consecutive times (9 A.M., 1 P.M., 9 P.M.) totaling the high dose (3x), the absorption exceeded that predicted from the single high dose.

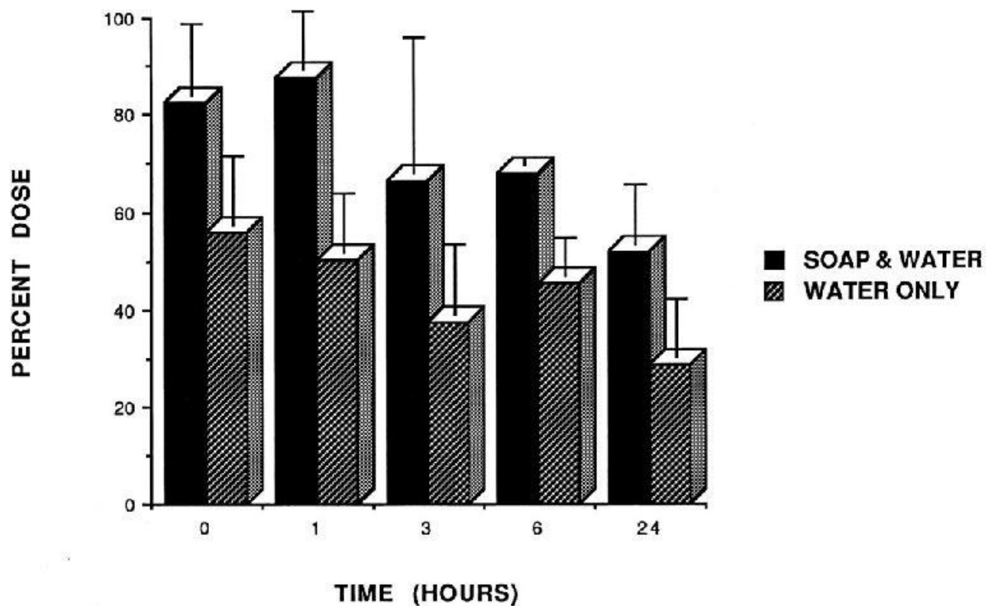

FIGURE 8 Skin decontamination of alachlor (lipophilic chemical) requires some soap to exceed removal by water only.

Figure 8 (alachlor) shows skin decontamination with soap and water or water only over a 24-hour dosing period, using the grid methodology. A series of 1 cm^2 areas are marked on the skin and each individual area is washed at a different time. Certain observations are made. First, the amount recovered decreased over time. This is because this is an in vivo system and percutaneous absorption is taking place, decreasing the amount of chemical on the skin surface. There also may be some loss attributable to skin desquamation. The second observation is that alachlor is more readily removed with soap-and-water wash than with water only. Alachlor is lipid soluble and needs the surfactant system for more successful decontamination [10].

Soap-and-water wash may not be the best method to cleanse skin. Soap and water will remove visible dirt and odor, but may not be a good skin cleanser. Figure 9 shows methylene bisphenyl isocyanate (MDI) (an industrial chemical) decontamination with water, soap and water, and some polyglycol and oil-based cleansers. Water and soap and water didn't work well but the polyglycol and oil-based cleansers did the job. The unknown question that remains is whether soap and water would then remove the polyglycol and oil-based cleansers [11].

COSMETIC PERCUTANEOUS ABSORPTION AND TOXICITY

The potential toxicity of cosmetics has in the past been dismissed as an event unlikely to occur. The argument was put forth that cosmetics did not contain ingredients that could prove harmful to the body. The argument went further to say that, because cosmetics were applied to skin with its barrier properties, the likelihood that a chemical would become systemically available was remote. The argument was proven false when carcinogens were

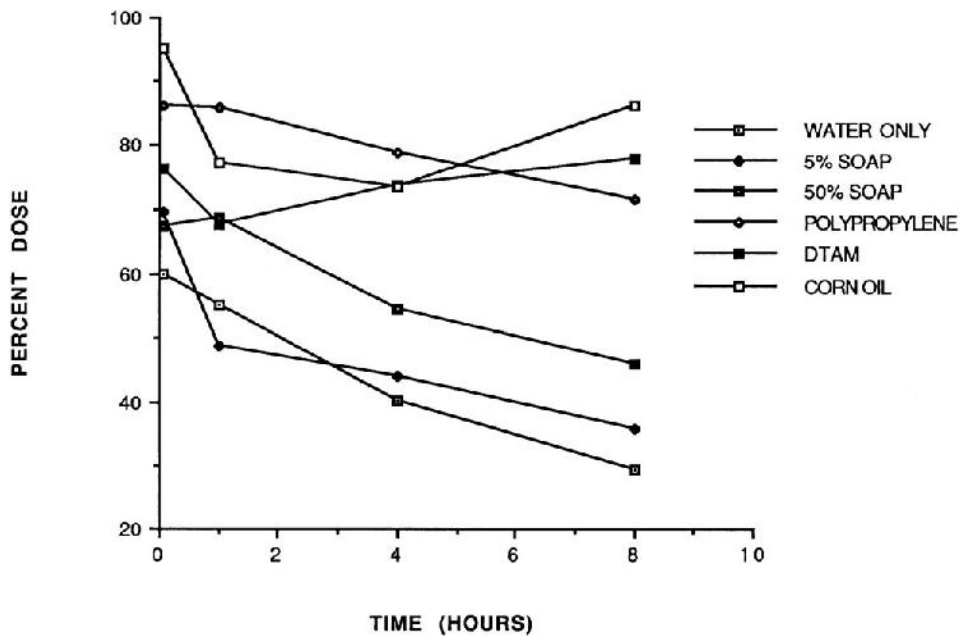

FIGURE 9 Methylene bisphenyl isocyanate (MDI) skin decontamination. Water alone and soap and water were relatively ineffective in removing MDI compared with the polypropylene-based decontaminants and corn oil.

shown to be present in cosmetics, and subsequent studies showed that these carcinogenic chemicals could be percutaneously absorbed [12].

Table 2 shows the relationship between percutaneous absorption and erythema for several oils used in cosmetics. The investigators attempted to correlate absorbability with erythema. The most-absorbed oil, isopropyl myristate, produced the most erythema. The lowest-absorbing oil, 2-hexyldecanoxyoctane, produced the least erythema. Absorbability and erythema for the other oils did not correlate [13]. The lesson to remember with percutaneous toxicity is that a toxic response requires both an inherent toxicity in the chemical and percutaneous absorption of the chemical. The degree of toxicity will depend on the contributions of both criteria.

In the rhesus monkey, the percutaneous absorption of safrole, a hepatocarcinogen,

TABLE 2 Relationship of Percutaneous Absorption and Erythema for Several Oils Used in Cosmetics

Absorbability (greatest to least)	Erythema
Isopropyl myristrate	++
Glycol tri(oleate)	−
n-Octadecane	±
Decanoxydecane	+
2-Hexyldecanoxyoctane	−

was 6.3% of applied dose. When the site of application was occluded, the percutaneous absorption doubled to 13.3%. Occlusion is a covering of the application site, either intentionally, as with a piece of plastic taped over the dosing site during experimentation, or unintentionally, as by putting on clothing after applying a cosmetic. The percutaneous absorption of cinnamic anthranilate was 26.1% of the applied dose, and this increased to 39.0% when the site of application was occluded. The percutaneous absorption of cinnamic alcohol with occlusion was 62.7%, and that of cinnamic acid with occlusion was 83.9% of the applied dose. Cinnamic acid and cinnamic aldehyde are agents that elicit contact urticaria [14], and cinnamic aldehyde is positive for both Draize and maximization methods [15,16].

In vivo human skin has the ability to metabolize chemicals. Figure 10 shows the metabolic profile of extracted human skin after pure hydroquinone had been dosed on the skin for 24 hours. The metabolic profile shows unchanged hydroquinone and its metabolite benzoquinone [3].

We have thus learned that common cosmetic ingredients can readily penetrate skin and become systemically available. If the cosmetic chemical has inherent toxicity, then that chemical will get into the body of a user and exert a toxic effect. Metabolically, the skin can also produce a more toxic compound.

The development of topical drug products requires testing for skin toxicology reactions. A variety of patch-test systems are available with which chemicals are applied to skin. A study was performed to determine the skin absorption of *p*-phenylenediamine (PPDA) from a variety of such systems. [^{14}C]PPDA (1% petrolatum UDP) was placed in a variety of patch-test systems at a concentration normalized to equal surface area (2 mg/

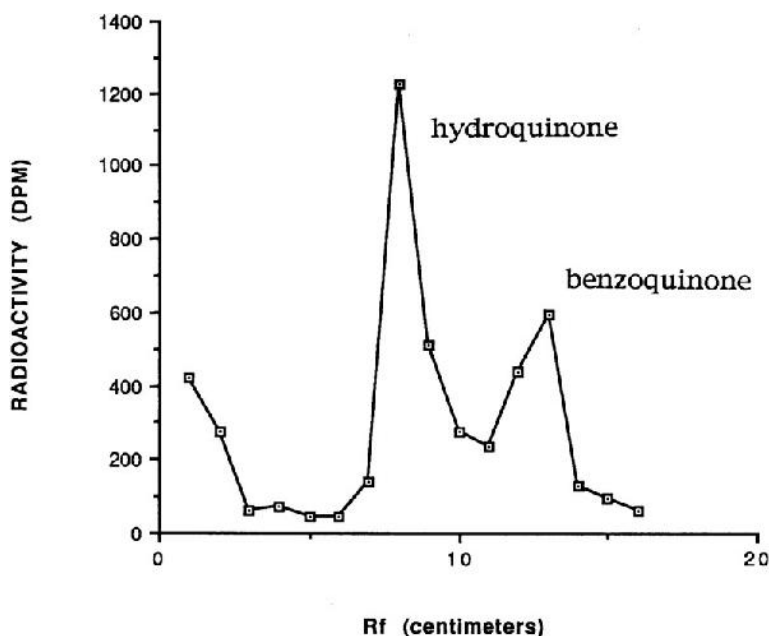

FIGURE 10 Hydroquinone dosed on viable skin was metabolically converted into the potential carcinogen benzoquinone within the human skin. The fate of a chemical within skin is more important than what is on the surface of skin.

TABLE 3 Percutaneous Absorption of *p*-Phenylenediamine (PPDA) from Patch-Test Systems

	Total load in chamber (mg)	Concentration in chamber (mg/mm²)	Absorption	
			Percent*	Total (mg)
Hill Top chamber	40	2	53.4 ± 20.6	21.4
Teflon (control)	16	2	48.6 ± 9.3	7.8
Small Finn chamber	16	2	29.8 ± 9.0	4.8
Large Finn chamber	24	2	23.1 ± 7.3	5.5
AL-test chamber	20	2	8.0 ± 0.8	1.6
Small Finn chamber with paper disc insert	16	2	34.1 + 19.8	5.5

* Each value is the mean + standard deviation for three guinea pigs.

mm^2). Skin absorption was determined in the guinea pig by urinary excretion of ^{14}C. There was a sixfold difference in the range of skin absorption ($p < 0.02$). In decreasing order, the percentage skin absorption from the systems were 53.4 ± 20.6 (Hill Top chamber), 48.6 ± 9.3 (Teflon control patch), 23.1 + 7.3 (small Finn chamber), and 8.0 + 0.8 (AL-test chamber). Thus, the choice of patch system could produce a false-negative error if the system inhibits skin absorption, with a subsequent toxicology reaction (Table 3) [17].

COSMECEUTICS

The early concept of cosmetics was one of inert ingredients used as coloring or cover agents to enhance visual appearance. There was no concern with systemic toxicity because skin had barrier properites and it was assumed nothing would permeate across the skin. The line between cosmetics and pharmaceutics has become a gray area as more active agents are incorporated into cosmetics. These active agents are referred to as *cosmeceutics*. Hydroquinone when prescribed by a physician is a drug. Hydroquinone in a cosmetic as a lightening agent is not a drug. The only differentiation between the two preparations is the hydroquinone concentration in the preparation. However, applied concentration does not matter; what matters is how much of the hydroquinone gets into and through the skin. For hydroquinone, percutaneous absorption is 45% of the applied dose for a 24-hour application to in vivo human skin [3]. That is a lot of drug—or is it cosmetic, or cosmeceutic? The important point is that for active chemicals the bioavailability needs to be known to assess risk assessment.

Another example is α-tocopherol, or vitamin E [18]. The biological activities of vitamin E in cosmetics are supported by several studies of its percutaneous absorption. In data obtained in vitro on rat skin 6 hours after application of a 5% vitamin E alcohol solution, 38.6% of the applied dose was recovered in the viable epidermis and dermis. The amount detected in the horny layer was 7.12%, and the residual fraction persisting on the surface on the integument represented 54.3% of the applied dose. Both the alcohol and acetate forms of vitamin E are readily absorbed through the human scalp, and within 6 to 24 hours after treatment they concentrate in the dermis. These results substantiate the claim that vitamin E can be used as an active ingredient in cosmetology with the possibility of efficacy in the deeper structures of the skin. Table 4 summarizes the in vitro percutaneous absorption of vitamin E acetate into and through human skin. Each

TABLE 4 In Vitro Percutaneous Absorption of Vitamin E Acetate Into and Through Human Skin

	Percent dose absorbed		
Treatment	Receptor fluid	Skin content	Surface wash
Formula A			
Skin source 1	0.34	0.55	74.9
Skin source 2	0.39	0.66	75.6
Skin source 3	0.47	4.08	89.1
Skin source 4	1.30	0.96	110.0
Mean + SD	0.63 + 0.45*	1.56 + 1.69†	87.4 + 16.4
Formula B			
Skin source 1	0.24	0.38	—
Skin source 2	0.40	0.64	107.1
Skin source 3	0.41	4.80	98.1
Skin source 4	2.09	1.16	106.2
Mean + SD	0.78 + 0.87*	1.74 + 2.06†	103.8 + 5.0

* $p = 0.53$ (nonsignificant; paired t-test).
† $p = 0.42$ (nonsignificant; paired t-test).

formulation was tested in four different human skin sources. The percent dose absorbed for a 24-hour dosing period is given for receptor-fluid accumulation (absorbed), skin content, and surface wash (soap-and-water wash recovery after the 24-hour dosing period).

Table 4 also contains what is referred to as *material balance*. All of the applied dose is accounted for in the receptor fluid, skin content, and skin-surface wash. Total absorbed dose would be the sum of that in the receptor fluid plus that in the skin (content). This is an example of a complete in vitro percutaneous absorption study.

DISCUSSION

The concepts of cosmetics and of the skin have undergone changes in the last few decades. Cosmetics have evolved from being formulations of inert ingredients to containing ingredients that have some biological activity directed to living skin. This is sometimes referred to as cosmeceutics. The concept of skin has evolved from an impenetrable barrier to one where percutaneous absorption does occur. Risk assessment requires a knowledge of percutaneous absorption so that health is not jeopardized. This applies to any topically applied chemical, be it cosmetic, pharmaceutic, industrial, or environmental.

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8

Principles and Mechanisms of Skin Irritation

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INTRODUCTION

In contrast to allergic contact dermatitis (ACD), irritant contact dermatitis (ICD) is the result of unspecified damage attributable to contact with chemical substances that cause an inflammatory reaction of the skin [1]. The clinical appearance of ICD is extremely variable. It is determined by the type of irritant and a dose-effect relationship [2]. The clinical morphology of acute irritant contact dermatitis as one side of the spectrum is characterized by erythema, edema, vesicles that may coalesce, bullae, and oozing. Necrosis and ulceration can be seen with corrosive materials. Clinical appearance of chronic ICD is dominated by redness, lichenification, excoriations, scaling, and hyperkeratosis.

Any site of skin may be affected. Most frequently the hands as human ''tools'' come into extensive contact with irritants, whereas most adverse reactions to cosmetics occur in the face because of the particular sensitivity of this skin region. Airborne ICD develops in uncovered skin areas, mostly in the face and especially the periorbital region after exposure to volatile irritants or vapor [3,4].

Despite their different pathogenesis, allergic and irritant contact dermatitis, particularly chronic conditions, show a remarkable similarity with respect to clinical appearance, histopathology [5,6], and immunohistology [7,8]. Therefore, ICD can be regarded as an exclusion diagnosis after negative patch testing. The histological pattern of chronic irritant contact dermatitis is characterized by hyper- and parakeratosis, spongiosis, exocytosis, moderate to marked acanthosis, and mononuclear perivascular infiltrates with increased mitotic activity [9,10].

MOLECULAR MECHANISMS OF SKIN IRRITANCY

As mentioned, striking clinical similarities exist between ICD and ACD, and even extensive immunostaining of biopsies does not allow discrimination between the two types of dermatitis [8].

In contrast to ACD, ICD lacks hapten-specific T-lymphocytes. The pathogenic pathway in the acute phases of ICD starts with the penetration of the irritant into the barrier, either activation or mild damage of keratinocytes, and release of mediators of inflammation with unspecific T-cell activation [11]. Epidermal keratinocytes play the crucial role in the

inflammation of ICD; they can be induced to produce several cytokines and provoke a dose-dependent leukocyte attraction [12]. The upregulation of certain adhesion molecules like α6 integrin or CD 36 is independent from the stimulus and not cytokine induced [13,14]. A number of agents and cytokines themselves are capable of mediating cytokine production in keratinocytes. IL-1 and TNF-α play a role as inflammatory cytokines, IL-8 and IP-10 are known to act as chemotaxins, and IL-6, IL-7, IL-15, GM-CSF, and TGF-alpha can promote growth. Other cytokines, such as IL-10, IL-12, and IL-18, are known to regulate humoral versus cellular immunity [15]. It is controversial whether the cytokine profile induced by irritants differs from that induced by allergens [16]. In irritant reactions, TNF-alpha, IL-6, IL-1β, and IL-2 have been reported to be increased [17,18].

In subliminal contact to irritants, barrier function of the stratum corneum and not the keratinocyte is the main target of the insulting stimulus. Damage of the lipid barrier of the stratum corneum is associated with loss of cohesion of corneocytes and desquamation with increase of transepidermal water loss (TEWL). This is one triggering stimulus for lipid synthesis and it promotes barrier restoration [19]. Nevertheless, recent studies show that the concept of TEWL increase after sodium lauryl sulfate (SLS) being directly related to a delipidizing effect of surfactants on the stratum corneum cannot be kept up without limitation. Fartasch et al. showed that SLS exposure for 24 hours causes damage in the deeper nucleated cells of the epidermis, leaving the lamellar arrangements of lipids intact. This means that the hypothetical model of SLS-induced irritation is mainly modulated by keratinocytes rather than the stratum corneum [20].

The stratum corneum influences epidermal proliferation after contact to irritants by increasing the mitotic activity of basal keratinocytes and in this way enhancing the epidermal turnover [21,22]. Disruption of the stratum corneum can even stimulate cytokine production itself and in this way promote the inflammatory skin reaction, as shown by Wood et al. [23]. They found an increase of TNF-α, various interleukins, and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Recently it has been shown that chemically different irritants induce differences in the response in the epidermis during the first 24 hours with respect to cytokine expression, indicating different ''starting points'' for the inflammatory response that results in the same irritant response clinically after 48 hours. Nonanionic acid, but not SLS, induced an increase in m-RNA expression for IL-6, whereas m-RNA expression for GM-CSF was increased after SLS [24]. Forsey et al. saw a proliferation of keratinocytes after 48 hours of exposure, and apoptosis of keratinocytes after 24 and 48 hours of exposure to SLS. In contrast, nonanionic acid decreased keratinocyte proliferation after 24 hours of exposure and epidermal cell apoptosis after only 6 hours of exposure [25]. In conclusion, it becomes clear that the concept of skin irritation is complicated and we are only beginning to understand the underlying molecular mechanisms.

FACTORS PREDISPOSING TO CUTANEOUS IRRITATION

The skin of different individuals differs in susceptibility to irritation in a remarkable manner, and a number of individual factors influencing development of irritant dermatitis that have been identified include age, genetic background, anatomical region exposed, and preexisting skin disease.

Although experimental studies did not support sex differences of irritant reactivity [26,27], females turned out to be at risk in some epidemiological studies [28,29]. It is probable that increased exposure to irritants at home, caring for children under the age

of 4 years, lack of dishwashing machine [30], and preference for high-risk occupations contribute to the higher incidence of ICD in females [27]. The most established individual risk factor, out of several studies about occupational hand eczema, is probably atopic dermatitis [28,31–33]. On the other hand, experimental studies concerning the reactivity of atopics and nonatopics to standard irritants have given contradictory results [34,35] and, as shown in a Swedish study, about 25% of the atopics in extreme-risk occupations, such as hairdressers and nursing assistants, did not develop hand eczema [36]. Age is as well related to irritant susceptibility insofar as irritant reactivity declines with increasing age. This is true not only for acute but also for cumulative irritant dermatitis [37,38]. Fair skin, especially skin type I, is supposed to be the most reactive to all types of irritants, and black skin is the most resistant [39,40].

Clinical manifestation of ICD is also influenced by type and concentration of irritant, solubility, vehicle, and length of exposure [41], as well as temperature and mechanical stress. During the winter months, low humidity and low temperature decrease the water content of the stratum corneum and increase irritant reactivity [42,43].

EPIDEMIOLOGY

Population-based data on the incidence and prevalence of ICD are rare, but there is agreement that incidence of ICD is higher than that of ACD in general. The figures on the incidence of ICD vary considerably, depending on the study population. Most data stem from studies about occupational hand dermatoses, and in this an overview is given about the important findings of these studies. In general, it can be assumed that nonoccupational contact dermatitis attributable to all causes is more frequent in comparison to occupational contact dermatitis [29].

Coenraads and Smit reviewed international prevalence studies for eczema attributable to all causes conducted with general populations in different countries (England, The Netherlands, Norway, Sweden, the United States) and found point prevalence rates of 1.7 to 6.3%, and 1- to 3-year period prevalence rates of 6.2 to 10.6% [44].

An extensive study of Meding on hand eczema in Gothenburg, Sweden, included 20,000 individuals randomly selected from the population register [28]. She estimated a 1-year period prevalence of hand eczema of 11% attributable to all causes, and a point prevalence of 5.4%. ICD contributed to 35% of the cases, whereas 22% were diagnosed as atopic hand dermatitis and 19% as ACD. In a multicenter epidemiological study on contact dermatitis in Italy by GIRDCA (Gruppo Italiano Ricerca Dermatiti da Contatto e Ambientali) 42,839 patients with contact dermatitis underwent patch testing. In accordance with the findings of Meding, nonoccupational as well as occupational ICD affected women in a higher percentage compared with males [28,29]. In Heidelberg, Germany, a retrospective study of 190 cases of hand dermatitis revealed 27% as ICD, 15,8% as ACD, and the majority (40%) as being of atopic origin with 10% various other diseases [45].

Shenefelt studied the frequency of visits by university students to campus prepaid–health-plan dermatologists for irritant and allergic contact dermatitis compared with other types of dermatitis and skin problems. In contrast to other studies, he found slightly more cases of allergic (3.1% of all first visits) than irritant contact dermatitis (2.3%) [46].

Reports on adverse reactions to cosmetics, including those with only subjective perceptions without morphological signs, are more frequent than assumed. In a questionnaire carried out in Thuringia, eastern Germany, even 36% of 208 persons reported adverse cutaneous reactions against cosmetics, 75% of them being female [47]. Nevertheless, it

must be emphasized that this includes, in addition to allergic contact dermatitis, dermatoses as seborrheic dermatitis, perioral dermatitis, rosacea and psoriasis, which cannot be separated by the unexperienced. Higher incidence in females was confirmed by several studies [48]. Most untoward reactions caused by cosmetics occur on the face, including the periorbital area [49].

In a study by Broeckx et al., 5.9% of a test population of 5202 patients with possible contact dermatitis had adverse reactions to cosmetics. Patch testing classified only 1.46% as irritant reactions whereas 3.0% could be classified as ACD. More than 50% of the cases of irritation were attributable to soaps and shampoos [50]. In Sweden, the top-ranking products causing adverse effects, as reported by the Swedish Medical Products Agency, were moisturizers, haircare products, and nail products [48].

In other studies, the incidence of cosmetic intolerance varied between 2 and 8.3%, depending on the test population [49,51,52]. In a large multicenter prospective study on reactions caused by cosmetics, Eiermann et al. found irritancy to account for only 16% of 487 cases of contact dermatitis caused by cosmetics. Of 8093 patients tested for contact dermatitis, 487 cases (6%) were diagnosed as contact dermatitis caused by cosmetics [53]. Since most consumers just stop using cosmetics when experiencing mild irritant or adverse reactions and seldom consult a physician, it can be assumed that mild irritant reactions to cosmetic products are underestimated [54].

CLINICAL TYPES OF IRRITANT CONTACT DERMATITIS

According to the highly variable clinical picture, several different forms of ICD have been defined. The following types of irritation have been described [55,56]:

- Acute ICD
- Delayed acute ICD
- Irritant reaction
- Cumulative ICD
- Traumiterative ICD
- Exsiccation eczematid
- Traumatic ICD
- Pustular and acneiform ICD
- Nonerythematous
- Sensory irritation

Acute ICD

Acute ICD is caused by contact to a potent irritant. Substances that cause necrosis are called corrosive and include acids and alkaline solutions. Contact is often accidental at the workplace. Cosmetics are unlikely to cause this type of ICD because they do not contain primary irritants in sufficient concentrations.

Symptoms and clinical signs of acute ICD develop with a short delay of minutes to hours after exposure, depending on the type of irritant, concentration, and intensity of contact. Characteristically the reaction quickly reaches its peak and then starts to heal; this is called ‘‘decrescendo phenomenon.’’ Symptoms include burning rather than itching,

stinging, and soreness of the skin, and are accompanied by clinical signs such as erythema, edema, bullae, and even necrosis. Lesions are usually restricted to the area that came into contact, and sharply demarcated borders are an important sign of acute ICD. Nevertheless, clinical appearance of acute ICD can be highly variable and sometimes may even be indistinguishable from the allergic type. In particular, combination of irritant and allergic contact dermatitis can be troublesome. Prognosis of acute ICD is good if irritant contact is avoided.

Delayed Acute ICD

For some chemicals, such as anthralin, it is typical to produce a delayed acute ICD. Visible inflammation is not seen until 8 to 24 hours or more after exposure [57]. Clinical picture and symptoms are similar to acute ICD. Other substances that cause delayed acute ICD include dithranol, tretinoin, and benzalkonium chloride. Irritation to tretinoin can develop after a few days and results in a mild to fiery redness followed by desquamation, or large flakes of stratum corneum accompanied by burning rather than itching. Irritant patch-test reactions to benzalkonium chloride may be papular and increase with time, thus resembling allergic patch-test reactions [58]. Tetraethylene glycol diacrylate caused delayed skin irritation after 12 to 36 hours in several workers in a plant manufacturing acrylated chemicals [59].

Irritant Reaction

Irritants may produce cutaneous reactions that do not meet the clinical definition of a ''dermatitis.'' Irritant reaction is therefore a subclinical form of irritant dermatitis and is characterized by a monomorphic rather than polymorphic picture. This may include one or more of the following clinical signs: dryness, scaling, redness, vesicles, pustules, and erosions [60]. Irritant reactions often occur after intense water contact and in individuals exposed to wet work, such as hairdressers or metal workers, particularly during their first months of training. It often starts under rings worn on the finger or in the interdigital area, and may spread over the dorsum of the fingers and to the hands and forearms. Frequently, the condition heals spontaneously, resulting in hardening of the skin, but it can progress to cumulative ICD in some cases.

Cumulative ICD

Cumulative ICD is the most common type of ICD [55]. In contrast to acute ICD that can be caused by single contact to a potent irritant, cumulative ICD is the result of multiple subthreshold damage to the skin when time is too short for restoration of skin-barrier function [61]. Clinical symptoms develop after the damage has exceeded a certain manifestation threshold, which is individually determined and can vary within one individual at different times. Typically, cumulative ICD is linked to exposure of several weak irritants and water contact rather than to repeated exposure to a single potent irritant. Because the link between exposure and disease is often not obvious to the patient, diagnosis may be considerably delayed, and it is important to rule out an allergic cause. Symptoms include itching and pain caused by cracking of the hyperkeratotic skin. The clinical picture is dominated by dryness, erythema, lichenification, hyperkeratosis, and chapping. Xerotic dermatitis is the most frequent type of cumulative toxic dermatitis [62]. Vesicles are less

frequent in comparison to allergic and atopic types [28]; however, diagnosis is often complicated by the combination of irritation and atopy, irritation and allergy, or even all three. Lesions are less sharply demarcated in contrast to acute ICD.

Prognosis of chronic cumulative ICD is rather doubtful [63,64]. Some investigators suggest that the repair capacity of the skin may enter a self-perpetuating cycle [61].

Traumiterative ICD

This term is often used similarly to cumulative ICD [55,60]. Clinically, the two types are very similar as well. According to Malten and den Arend, traumiterative ICD is a result of too-early repetition of just one type of load, whereas cumulative ICD results from too-early repetition of different types of exposures [2].

Exsiccation Eczematid

Exsiccation eczematid is a subtype of ICD that mainly develops on the extremities. It is often attributable to frequent bathing and showering as well as extensive use of soaps and cleansing products. It often affects elderly people with low sebum levels of the stratum corneum. Low humidity during the winter months and failure to remoisturize the skin contribute to the condition. The clinical picture is typical, with dryness, ichthyosiform scaling, and fissuring. Patients often suffer from intense itching.

Traumatic ICD

Traumatic ICD may develop after acute skin traumas such as burns, lacerations, and acute ICD. The skin does not heal as expected, but ICD with erythema, vesicles and/or papulovesicles, and scaling appears. The clinical course resembles that of nummular dermatitis [55].

Pustular and Acneiform ICD

Pustular and acneiform ICD may result from contact to irritants such as mineral oils, tars, greases, some metals, croton oil, and naphthalenes. Pustules are sterile and transient. The syndrome must be considered in conditions in which acneiform lesions develop outside typical acne age. Patients with seborrhoea, macroporous skin, and prior acne vulgaris are predisposed along with atopics.

Nonerythematous ICD

Nonerythematous ICD is an early stage of skin irritation that lacks visible inflammation but is characterized by changes in the function of the stratum corneum that can be measured by noninvasive bioengineering techniques [55,65].

Sensory Irritation

Sensory irritation is characterized by subjective symptoms without morphological changes. Predisposed individuals complain of stinging, burning, tightness, itching, or even painful sensations that occur immediately or after contact. Those individuals with hyperirritable skin often report adverse reactions to cosmetic products with most reactions occurring on the face. Fisher defined the term ''status cosmeticus,'' which describes a condition in patients who try a lot of cosmetics and complain of being unable to tolerate any

of them [66]. Lactic acid serves as a model irritant for diagnosis of so called ''stingers'' when it is applied in a 5% aqueous solution on the nasolabial fold after induction of sweating in a sauna [67]. Other chemicals that cause immediate-type stinging after seconds or minutes include chloroform and methanol (1:1) and 95% ethanol. A number of substances that have been systematically studied by Frosch and Kligman may also cause delayed-type stinging [67,68]. Several investigators tried to determine parameters that characterize those individuals with sensitive skin, a term that still lacks a unique definition [69,70]. It could be shown that individuals who were identified as having sensitive skin by their own assessment have altered baseline biophysical parameters, showing decreased capacitance values, increased transepidermal water loss, and higher pH values accompanied by lower sebum levels [70]. Possible explanations for hyperirritability (other than diminished barrier function) that have been discussed are heightened neurosensory input attributable to altered nerve endings, more neurotransmitter release, unique central information processing or slower neurotransmitter removal, and enhanced immune responsiveness [69,71]. It is not clear whether having sensitive skin is an acquired or inherited condition; most probably it can be both. As in other forms of ICD, seasonal variability in stinging with a tendency to more intense responses during winter has been observed [72]. Detailed recommendations for formulation of skincare products for sensitive skin have been given by Draelos [69].

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9

Allergy and Hypoallergenic Products

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INTRODUCTION

The assessment and detection of the number of contact allergic reactions to cosmetics are not simple. Generally, a consumer who has a problem with cosmetics will consult a doctor only if he or she does not recognize the cause to be a particular cosmetic product or if the dermatitis persists when the suspect product has been replaced by another, determined by trial and error. Consequently, only a small proportion of the population with cosmetic intolerance problems is ever seen by a dermatologist. Moreover, cosmetic reactions may present in unusual clinical forms, which may evoke an erroneous diagnosis [1–3].

In general, adverse effects are underreported [4], certainly to the cosmetics industry which obtains its most reliable information in this regard mainly from the relatively few dermatologists who concentrate on cosmetic-intolerance problems and from reports in the literature which are, almost by definition, out of date. Sometimes beauticians and consumers report adverse reactions, but in most cases this kind of information is difficult to objectify unless it is verified by a dermatologist.

Application of cosmetic products to the skin may cause irritant, phototoxic, contact, and photocontact allergic reactions as well as contact urticaria. It is generally agreed that most skin-adverse reactions to cosmetic products are irritant in nature and that people with ‘‘sensitive skin,’’ as indicated by conditions like atopic dermatitis, rosacea, or seborrheic dermatitis, are particularly liable to develop such reactions. However, contact allergic reactions attract much more attention and thus tend to be overestimated [4]. Indeed, the identification of the cosmetic allergen is by no means a simple task. It demands special skills and interest on the part of the dermatologist, even though the labeling of all cosmetic ingredients, which is now obligatory also in Europe, is facilitating that task. Moreover, there are many factors involved in the sensitization to a specific cosmetic product, all of which have to be taken into account when one seeks an allergen [1,2] (see the following section).

FACTORS CONTRIBUTING TO CONTACT ALLERGIC REACTIONS TO A COSMETIC PRODUCT

Frequency of Use

One may expect frequently used products to cause more skin reactions than more exclusive products simply because more people are exposed to them. This alone does not imply anything about the quality of these products (the same thing may be said about individual cosmetic ingredients).

Composition

The complexity of a formula can be either positive or negative as far as its allergenicity is concerned. One of the principles of creating ''hypoallergenic'' cosmetics and perfumes is simplicity of formula. The fewer the constituents, the easier it is to identify the offending substance should difficulties arise, and the less danger there is of synergism. The more ingredients there are, the more chance there is of sensitization by one of them. However, some investigators recommend placing upper limits on concentrations rather than advising against the use of any particular ingredient. They may also suggest more complex formulas [5].

Preservatives are needed in water-based or other easily contaminated products and are common cosmetic allergens. It seems that it is very difficult to combine potent antimicrobial and antifungal properties with low allergenicity. Indeed, it is very difficult to restrict the biological activity of a substance to a single domain.

Concentration of Ingredients

Although the use of low concentrations does not assure complete safety, the incidence of sensitization induction is, indeed, a function of the concentration of the allergen, at least to some extent. Cases of allergy to the preservative agent (chloro)methylisothiazolinone illustrate this problem very well. At first, when a 50 ppm concentration of this agent was allowed for use in cosmetic products in the European Community and when this concentration was actually used in some products, there were ''epidemics'' of contact allergic reactions to it [6]. Of late, the frequency of positive reactions has been diminishing considerably, not only because its use is declining and primarily limited to ''rinse-off'' products [3] but also because its usage concentration has been reduced to 15 to 7.5 ppm (as the manufacturers recommended). Of course, once a patient has become sensitized, even low concentrations can trigger a reaction.

Purity of Ingredients

It is impossible to refine raw materials to absolute purity. More or less strict quality control of raw materials and finished products has long been general practice in modern cosmetic manufacturing. However, one can never rule out the sensitizing potential of impurities in these materials [5].

The Common Use of Cosmetic Ingredients in Pharmaceuticals

Patients easily become sensitized to topical pharmaceutical products which, unlike cosmetics, are most often used on diseased skin. Once sensitization has occurred, however, they may react to cosmetics containing the same ingredients [5].

The Role of Cross-Sensitivity

Chemically related substances are likely to induce cross-reactions and contact eczematous lesions may be maintained in this way. This is especially the case with perfume ingredients, which often cross-react with each other, but applies to all other cosmetic ingredients as well.

Penetration-Enhancing Substances

The chemical environment can substantially affect the sensitizing potential of individual chemicals. For example, emulsifiers and solvents enhance skin penetration and thereby contact sensitization. Penetration-enhancing agents can also be the root of false-negative patch-test reactions; the cosmetic product itself may be clearly allergenic (or irritant) although the individual ingredients, abstracted from the environment of the product and tested separately, may not cause a reaction.

Application Site

Some areas of the skin, like the eyelids, are particularly prone to contact dermatitis reactions. A cream applied to the entire face such as a facecare product, along with hair products may cause an allergic reaction only on the eyelids. Moreover, ‘‘ectopic dermatitis’’ [caused by the transfer of the allergen by the hand, as often occurs with tosylamide/formaldehyde (= para-toluenesulfonamideformaldehyde) resin, the allergen in nail polish], ‘‘airborne’’ contact dermatitis (e.g., caused by perfumes) [7], as well as ‘‘connubial’’ dermatitis (caused by products used by the partner) [8] often occur only on ‘‘sensitive’’ skin areas such as the eyelids, the lips, and the neck.

Moreover, the penetration potential of cosmetics is heightened in certain ‘‘occluded’’ areas, such as the body folds (axillary, inguinal) and the anogenital region, which also increases the risk of contact sensitization. In the body folds, the allergenic reactions tend to persist for weeks after the initial contact with the allergen. This may be partly attributable to residual contamination of clothing as well as the increased penetration of the allergen, which is certainly assisted by occlusion and friction [9]. Indeed, a reservoir may be formed from which the allergen is subsequently released.

Condition of the Skin

Application on damaged skin, where the skin barrier is impaired, enhances the penetration of substances and thus increases the risk of an allergic reaction. This is the case with bodycare products used to alleviate dry, atopic skin and with barrier creams for protecting the hands, which often suffer from irritancy problems (e.g., dryness, cracking). Sometimes, the allergic reaction may be limited to certain areas of the skin (areas already affected react more readily to another application of the same allergen) and may even present an unusual clinical picture that does not immediately suggest contact dermatitis. Indeed, contact allergic reactions to preservative agents on the face may present as a lymphocytic infiltrate or even have a lupus erythematous–like picture [3,10].

Contact Time

In the world of cosmetics, a distinction is now being made between leave-on products, which remain on the skin for several hours (e.g., face- and bodycare products and makeup), and rinse-off products, which are removed almost immediately.

The division between these two kinds of products is not always relevant to the sensitization process because a thin film can remain on the skin and be sufficient to allow ingredients to penetrate. This occurs, for example, with moist toilet paper (with mainly preservatives as the allergens) and makeup removers.

Frequency of Application and Cumulative Effects

Daily use or use several times a day of cosmetics may cause ingredients to accumulate in the skin and thus increase the risk of adverse reactions. In fact, the concentration of an ingredient may be too low to induce sensitivity in a single product but may reach critical levels in the skin if several products containing it are used consecutively. This may be the case for people who are loyal to the same brand of, e.g., day and night creams, foundations, and cleansing products, because a manufacturer will often use the same preservative system for all of its products. This should be taken into consideration by companies that use biologically active ingredients such as preservative agents, emulsifiers, antioxidants, and perfumes, because it might well account for many of the adverse reactions to these particular substances. In our experience, intense users of cosmetics are more prone to cosmetic dermatitis than others.

CORRELATIONS WITH THE LOCATION OF THE LESIONS

Like many other contact allergens, cosmetics can reach the skin in several different ways [1,2]: by direct application; by airborne exposure to vapors, droplets, or particles that are released into the atmosphere and then settle on the skin [7]; by contact with people (partners, friends, coworkers) who transmit allergens to cause ''connubial'' or ''consort'' dermatitis [8]; by transfer from other sites on the body, often the hands, to more sensitive areas such as the mouth or the eyelids (ectopic dermatitis); and by exposure to the sun with photoallergens.

The most common sources of cosmetic allergens applied directly to the body are listed in Table 1.

THE NATURE OF COSMETIC ALLERGENS

Fragrance Ingredients

Fragrance ingredients are the most frequent culprits in cosmetic allergies [11–15]. Katsarar et al., who investigated the results of patch testing over a 12-year period, found an increasing trend in sensitivity to fragrance compounds, which reflects the effectiveness of the advertising of perfumed products [16]. Common features of a fragrance contact dermatitis are localization in the axillae, localization on the face (including the eyelids) and neck, and well-circumscribed patches in areas of dabbing-on perfumes (wrists, behind the ears) and hand eczema or its aggravation. Airborne or connubial contact dermatitis should be considered as well.

Other less frequent adverse reactions to fragrances are photocontact dermatitis, contact urticaria, irritation, and pigmentation disorders [17].

Sensitization is most often induced by highly perfumed products, such as toilet waters, aftershave lotions, and deodorants, the last of which have recently been shown to contain well-known allergens such as cinnamic aldehyde and iso-eugenol [18].

TABLE 1 Cosmetic and Cosmetic-Related Dermatitis Caused by Direct Application of the Allergen

Area of dermatitis	Cosmetics that may contain allergens
Face in general	Facial skincare products (creams, lotions, masks), sunscreen products, makeup (foundations, blushes, powders), cleansers (lotions, emulsions), and cosmetic appliances (sponges), perfumed products (after-shave lotion)
Forehead	Haircare products (dyes, shampoos)
Eyebrows	Eyebrow pencil, depilatory tweezers
Upper eyelids	Eye makeup (eye shadow, eye pencils, mascara), eyelash curlers
Lower eyelids	Eye makeup
Nostrils	Perfumed handkerchiefs
Lips, mouth, and perioral area	Lipstick, lip pencils, dental products (toothpaste, mouthwash), depilatories
Neck and retroauricular area	Perfumes, toilet waters, haircare products
Head	Haircare products (hair dyes, permanent-wave solutions, bleaches, shampoo ingredients), cosmetic appliances (metal combs, hairpins)
Ears	Haircare products, perfume
Trunk/upper chest, arms, wrists (elbow flexures)	Bodycare products, sunscreens and self-tanning products, cleansers, depilatories
Axillae	Deodorants, antiperspirants, depilatories
Anogenital areas	Deodorants, moist toilet paper, perfumed pads, depilatories
Hands	Handcare products, barrier creams, all cosmetic products that come in contact with the hands
Feet	Footcare products, antiperspirants

As reported in the literature, the fragrance mix remains the best screening agent for contact allergy to perfumes because it detects some 70 to 80% of all perfume allergies [19,20]. However, additional perfume-allergy markers are certainly needed.

Preservatives

Preservatives are second in frequency to fragrance ingredients; they are important allergens in cleansers, skincare products, and makeup [12,21]. However, within this class important shifts have occurred over the years.

The methyl(chloro)isothiazolinone mixture was commonly used in the 1980s and was then a frequent cause of contact allergies. This frequency has declined considerably in recent years [3,12]. Since then, formaldehyde and its releasers—particularly methyldibromoglutaronitrile (=dibromodicyanobutane) as used in a mixture with phenoxyethanol, better known as EUXYL K400—did gain in importance in this regard [12,21–25], although the frequency of positive reactions observed seems to be influenced by the patch-test concentration [24,25].

The spectrum of the allergenic preservatives also varies from country to country. For example, in contrast to continental Europe where reactions to methyl(chloro)isothiazolinone and more recently methyldibromoglutaronitrile have been the most frequent, [12,13,21,26], in the United Kingdom formaldehyde and its releasers have always been

much more important, particularly as concerns quaternium-15 [21] although its incidence seems to have recently slightly decreased [27]. Parabens are rare causes of cosmetic dermatitis. When a paraben allergy does occur, the sensitization source is most often a topical pharmaceutical product, although its presence in other products can be sensitizing as well [28]. Recently, we observed such a case (data on file): a young lady, after having previously been sensitized to mefenesin in a rubefacient, presented with an acute contact dermatitis on the face at the first application of a new cosmetic cream containing chlorphenesin, which was used as a preservative agent. Apparently it is a potential sensitizing agent [29] and probably cross-reacts with mefenesin, which is used in pharmaceuticals.

Antioxidants

Antioxidants form only a minor group of cosmetic allergens. Examples are propyl gallate, which may cross-react with other gallates and are also used as food additives, and t-butyl hydroquinone, a well-known allergen in the United Kingdom but not in continental Europe [21].

''Active'' or Category-Specific Ingredients

With regard to ''active'' or category-specific ingredients, in contrast to de Groot [3] we found an increase of the number of reactions to oxidative hair dyes (PPD and related compounds) during the period 1991–1996 compared with the period 1985–1990 [12,13]. According to one cosmetic manufacturer (personal communication, L'Oréal, 1997), the use of such hair dyes has more than doubled in recent years. However, the replacement since 1987 of PPD-hydrochloride by PPD-base—a more appropriate screening agent for PPD-allergy—may also have influenced the incidence [30]. They are important causes of professional dermatitis in hairdressers, who also often react to allergens in bleaches (persulfates, also causes of contact urticaria), permanent-wave solutions (primarily glycerylmonothioglycolate, which may provoke cross-sensitivity to ammoniumthioglycolate), and sometimes shampoos (e.g., cocamidopropylbetaine and formaldehyde) [31,32]. Sodium pyrosulfite (or metabisulfite), present in oxidative hair dyes (data on file), was recently also found to be a professional allergen.

Tosylamide/formaldehyde (=toluenesulfonamide formaldehyde) resin is considered an important allergen [4] and is the cause of ''ectopic'' dermatitis attributable to nail lacquer, which may also contain epoxy and (meth)acrylate compounds [3]. It often gives rise to confusing clinical pictures and may mimic professional dermatitis [33].

(Meth)acrylates are also causes of reactions to artificial nail preparations, more recently to gel formulations, in both manicurists and their clients [34].

Moreover, some more recently introduced ''natural'' ingredients may induce contact-allergic reactions. Some examples are butcher broom (*Ruscus aculateus*), which is also a potential allergen in topical pharmaceutical products [35], hydrocotyl (asiaticoside) [36], and dexpanthenol [37]. Farnesol, a well-known perfume ingredient and cross-reacting agent to balsam of Peru, has become a potential allergen in deodorants in which it is used for its bacteriostatic properties [38].

Some sunscreen agents such as benzophenone-3, which may also cause contact urticaria, and dibenzoylmethane derivatives have been recognized in the past as being important allergens [3,21,39–41]. Indeed, isopropyldibenzoylmethane was even withdrawn for this reason [3]. Methylbenzylidene camphor, cinnamates, and phenylbenzimidazole sul-

fonic acid are only occasional, sometimes even rare, causes of cosmetic reactions. The use of para-aminobenzoic acid (PABA) and its derivatives has decreased considerably. Contact allergic reactions to them were generally related to their chemical relationship to para-amino compounds [42], although they were also important photosensitizers [39].

In our experience [12,13,21], the contribution of sunscreens to cosmetic allergy is relatively small despite the increase in their use because of media attention being given to the carcinogenic and accelerated skin-aging effects of sunlight. The low rate of allergic reactions observed may well be because a contact allergy or a photoallergy to sunscreen products is often not recognized, since a differential diagnosis with a primary sun intolerance is not always obvious. Furthermore, the patch-test concentrations generally used might be too low [43], in part because of the risk of irritancy.

Excipients and Emulsifiers

Many excipients and emulsifiers are common ingredients to topical pharmaceutical and cosmetic products, the former being likely to induce sensitization. Typical examples are wool alcohols, fatty alcohols (e.g., cetyl alcohol), and propylene glycol [13]. They may also be sensitizing in cosmetics, as is the case with maleated soybean oil [44]. Emulsifiers in particular have long been regarded as irritants, but their sensitization capacities should not be overlooked. It is imperative, of course, that patch testing be properly performed to avoid irritancy and that the relevance of the positive reactions be determined. This is certainly the case for cocamidopropylbetaine, an amphoteric tenside mainly present in hair-and skin-cleansing products. Whether the compound itself or cocamidopropyl dimethylamine, an amido-amine, or dimethylaminopropylamine (both intermediates from the synthesis) are the actual sensitizers is still a matter of discussion [45,46]. It is also not clear whether cocamidopropyl-PG-dimonium chloride phosphate (phospholipid PTC) [47], a new allergen in skincare products, can cross-react with cocamidopropylbetaine.

Coloring Agents

Coloring agents other than hair dyes have rarely been reported as cosmetic allergens. However, with the increased use of cosmetic tattoos (e.g., eye and lip makeup), more treatment-resistant skin lesions might develop in the future [48].

DIAGNOSING COSMETIC ALLERGY

Taking the history of the patient and noting the clinical symptoms and localization of the lesions are critical. Allergen identification for a patient with a possible contact allergy to cosmetics is performed by means of patch testing with the standard series, specific cosmetic-test series, the product itself, and all its ingredients. We can only find the allergens we look for. For skin tests with cosmetic products the patients supply themselves, there are several guidelines [49]. Not only patch and photopatch tests but also semiopen tests, usage tests, or repeated open application tests (ROATs) may need to be performed to obtain a correct diagnosis.

HYPOALLERGENIC PRODUCTS

Most of the cosmetic industry is making a great effort to commercialize products that are the safest possible. Some manufacturers market cosmetics containing raw materials having

a ‘‘low’’ sensitization index or a high degree of purity, or from which certain components have been eliminated [5,50] (generally perfume ingredients). Sometimes ‘‘active’’ preservative agents are also omitted, and immunologically inert physical agents are being used more often in sunscreens rather than chemical ultraviolet (UV) absorbants.

Statements such as ‘‘recommended by dermatologists,’’ ‘‘allergy-tested,’’ or ‘‘hypoallergenic’’ have been put on packaging by manufacturers to distinguish their products from those of their competitors. Although there are several ways to reduce allergenicity [3], there are no governmentally mandated standards or industry requirements [51].

The latest trend is target marketing to people with hypersensitive skin—an often-used term for the shadowy zone between normal and pathological skin. These would be people with increased neurosensitivity (e.g., atopics), heightened immune responsiveness (e.g., atopic and contact allergic individuals), or a defective skin barrier, i.e., people with irritable skin such as atopics or those suffering from seborrheic dermatitis [52] or rosacea. This means that part of the cosmetic industry is moving more into the area of pathological skin and that certain products are in fact becoming drugs, often called cosmeceuticals. This has caused a great deal of regulatory concern [53,54] both in the United States and the European Union because it suggests some middle category between cosmetics and drugs that does not yet legally exist. In Japan, however, these products fall in the category of ‘‘quasidrugs.’’

The meaning of most such claims used nowadays is unclear both for the dermatologist [50–52] and the consumer, the latter being convinced that hypersensitive skin is allergic skin. It is the dermatologist’s task to diagnose the skin condition and to provide specific advice about the products that can safely be used. All such problems must be approached individually, not at least the contact allergic types because people sensitive to specific ingredients must avoid products containing them. Therefore, ingredient labeling, which is also now required in Europe, can be of tremendous help. Providing the allergic patient with a limited list of cosmetics that can be used is practical and effective [55].

CONCLUSION

The identification of cosmetic allergens is challenging because of the extreme complexity of the problem. This applies not only for the dermatologist who is trying to identify the culprit and advise his patient but also certainly for cosmetic manufacturers, who are extremely concerned about assuring the innocuousness of their products. Precise, current, and rapid information about adverse reactions to cosmetic products is critical in product design. Apparently, premarketing studies are unable to identify all the pitfalls. Therefore, the fruitful communication that is developing between dermatologists and cosmetic manufacturers must be encouraged. Sensitivity to cosmetics can never be totally avoided, but its incidence can be substantially reduced.

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10

Dermatological Problems Linked to Perfumes

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INTRODUCTION

Perfumes are so much a part of our culture that we take them for granted. However, if they were suddenly taken from us, society would suffer immeasurably. We do pay a price for their service, and part of that concerns dermatological and other medical reactions. Adverse reactions to fragrances in perfumes and in fragranced cosmetic products include allergic contact dermatitis, irritant contact dermatitis, photosensitivity, immediate contact reactions (contact urticaria), pigmented contact dermatitis [1] and (worsening of) respiratory problems [2]. In this chapter, the issue of allergic contact reactions is discussed. (For a full review of side effects of fragrances [and essential oils] see Ref. 3.) A recent book on beneficial and adverse reactions to fragrances also provides valuable information [4]. The history of fragrances has been well described [5,6].

ALLERGIC CONTACT DERMATITIS FROM FRAGRANCES

Epidemiology

Considering the extensive use of fragrances, the frequency of contact allergy to them is relatively small. In absolute numbers, however, fragrance allergy is common. In a group of 90 student nurses, 12 (13%) were shown to be fragrance allergic [7]. In a group of 1609 adult subjects, 196 (12%) reported cosmetic reactions in the preceding 5 years. Sixty-nine of these (35% of the reactors and 4.3% of the total population) attributed their reactions to products primarily used for their smell (deodorants, aftershaves, perfumes) [8]. In 567 unselected individuals aged 15 to 69 years, 6 (1.1%) were shown to be allergic to fragrances as evidenced by a positive patch test reaction to the fragrance mix (vide infra) [9].

In dermatitis patients seen by dermatologists, the prevalence of contact allergy to fragrances is between 6 and 14%; only nickel allergy occurs more frequently. When tested with 10 popular perfumes, 6.9% of female eczema patients proved to be allergic to them [10] and 3.2 to 4.2% were allergic to fragrances from perfumes present in various cosmetic products [11]. In cosmetics causing contact allergic reactions, perfumes account for up to 18% and deodorants/antiperspirants for up to 17% of all cases. When patients with

suspected allergic cosmetic dermatitis are investigated, fragrances are identified as the most frequent allergens, not only in perfumes, aftershaves, and deodorants, but also in other cosmetic products not primarily used for their smell [12–15].

Patients allergic to fragrances are usually adult individuals of either sex. They mainly become allergic by the use of cosmetics and personal-care products; occupational contact with fragrances is seldom important, not even in workers in the cosmetics industry [3].

Clinical Picture of Contact Allergy to Fragrances

Contact allergy to fragrances usually causes dermatitis of the hands, face, and/or armpits [16–18], the latter site being explained by contact allergy to deodorants and fragranced antiperspirants. In the face, the skin behind the ears and neck is exposed to high concentrations of fragrances in perfumes and aftershaves. Microtraumata from shaving facilitates (photo)contact allergy to aftershave fragrances. The sensitive skin of the eyelids is particularly susceptible to developing allergic contact dermatitis to fragrances in skincare products, decorative cosmetics, and cleansing preparations, as well as from fragrances spread through the air (airborne contact dermatitis) [19]. Most reactions are mild and are characterized by erythema (redness) only with some swelling of the eyelids. More acute lesions with papules, vesicles, and oozing may sometimes be observed. Dermatitis attributable to perfumes or toilet water tends to be ''streaky.'' In some cases, the eruption resembles other skin diseases such as nummular eczema, seborrhoeic dermatitis, sycosis barbae, or lupus erythematosus [20]. Lesions in the skin folds may be mistaken for atopic dermatitis. Psoriasis of the face may be induced or worsened by allergic contact dermatitis from fragrances. Hand eczema is also common in fragrance-sensitive patients [17,18]. However, fragrances are rarely the sole cause of hand eczema. Usually, patients first have irritant dermatitis or atopic dermatitis, which is later complicated by contact allergy to products used for treatment (fragranced topical drugs) or prevention (hand creams and lotions) of hand dermatitis, or to other perfumed products in the household, recreation, or work environment.

The Causative Products

Patients appear to become sensitized to fragrances especially by the use of deodorant sprays and/or perfumes, and to a lesser degree by cleansing agents, deodorant sticks, or hand lotions [21]. Thereafter, new rashes may appear or are worsened by contact with other fragranced products: cosmetics, toiletries, oral hygiene products, household products, industrial contacts (e.g., cutting fluids, electroplating fluids, paints, rubber, plastics, additives in air-conditioning water), paper and paper products, laundered fabrics and clothes, topical drugs, and fragrances used as spices in foods and drinks [22]. By their ubiquitous use, virtually everyone is in daily contact with fragrance materials, which are very hard to completely avoid [3].

The Fragrance Allergens

Over 100 fragrances have been identified as allergens [3]. Most reactions are caused by the eight fragrances in the perfume mix (vide infra), and of these oak moss, isoeugenol, and cinnamic aldehyde (cinnamal) are the main sensitizers. Other fragrances (and essential

TABLE 1 Fragrances and Essential Oils That May Cause Contact Allergy in >1% of Patch-Tested Dermatitis Patients

α-amylcinnamic aldehyde	jasmine absolute
benzyl salicylate	jasmine synthetic
cananga oil	lilial
cinnamic alcohol	majantol
cinnamic aldehyde	methoxycitronellal
citral	methyl heptine carbonate
coumarin	methyl salicylate
dehydro-isoeugenol (in ylang-ylang oil)	musk ambrette
dihydrocoumarin	narcissus oil
eugenol	oak moss absolute
geraniol	oil of bergamot
geranium oil	patchouli oil
hydroabietyl alcohol	rose oil
hydroxycitronellal	sandalwood oil
isobornyl cyclohexanol (synthetic sandalwood)	sandela
isoeugenol	santalol
	ylang-ylang oil

Source: Refs. 3, 22.

oils used as fragrances) that cause contact allergy more than occasionally (>1% positive patch-test reactions in dermatitis patients routinely tested) are listed in Table 1.

The Diagnosis of Contact Allergy to Fragrances

Contact allergy to a particular product or chemical is established by means of patch testing. A perfume may contain as many as 200 or more individual ingredients. This makes the diagnosis of perfume allergy by patch-test procedures complicated. The fragrance mix, or perfume mix, was introduced as a screening tool for fragrance sensitivity in the late 1970s. It contains eight commonly used fragrances: α-amylcinnamic aldehyde, cinnamic alcohol, cinnamic aldehyde (cinnamal), eugenol, geraniol, hydroxycitronellal, isoeugenol, and oak moss absolute. It is estimated that this mix detects 70 to 80% of all cases of fragrance sensitivity [23]; this may be an overestimation because it was positive in only 57% of patients who were allergic to popular commercial fragrances [10]. The response rate to the fragrance mix in dermatological patients nowadays ranges worldwide from 6 to 14% [3,24]; only nickel sulphate yields more positive reactions.

In the United States, cinnamic aldehyde is routinely tested and scores 2.4% positive reactions [24]. In cases of suspected allergic cosmetic dermatitis, patients' personal products are always tested and may give positive patch-test reactions, proving that the patient is allergic to that product [18]. In addition, many investigators test (a series of) additional fragrances.

The fragrance mix is an extremely useful tool for the detection of cases of contact allergy to fragrances, but unfortunately is far from ideal: it misses 20 to 30% of relevant reactions or more, and may cause both false positive (i.e., a ''positive'' patch test reaction in a non–fragrance-allergic individual) and false negative (i.e., no patch test reaction in

an individual who is actually allergic to one or more of the ingredients of the mix) reactions [25].

Another useful test in cases of doubt (e.g., with weakly positive patch-test reactions that are difficult to interpret) is the repeated open application test (ROAT). The suspected allergen, which may be both an individual fragrance or scented product, is applied to the elbow flexure twice daily for a maximum of 14 days. A positive reaction confirms the existence of contact allergy and makes relevance of the reaction (vide infra) more likely.

The Relevance of Positive Patch Test Reactions to the Fragrance Mix

The finding of a positive reaction to the fragrance mix should be followed by a search for its relevance, i.e., if fragrance allergy is the cause of the patient's current or previous complaints or if it at least contributes to it. Often, however, correlation with the clinical picture is lacking and many patients can tolerate perfumes and fragranced products without problem [11]. This sometimes may be explained by irritant (false positive) patch-test reactions to the mix. Alternative explanations include the absence of relevant allergens in those products or a concentration too low to elicit clinically visible allergic contact reactions.

It is assumed that between 50 and 65% of all positive patch-test reactions to the mix are relevant, although this is sometimes hard to prove [24,26]. Nevertheless, there is a highly significant association between the occurrence of self-reported visible skin symptoms to scented products earlier in life and a positive patch test to the fragrance mix, and most fragrance-sensitive patients are aware that the use of scented products may cause skin problems [27].

In perfume-mix–allergic patients with concomitant positive reactions to perfumes or scented products used by them, interpretation of the reaction as relevant is highly likely. In such patients the incriminated cosmetics very often contain fragrances present in the mix, and thus the fragrance mix appears to be a good reflection of actual exposure [18]. Indeed, one or more of the ingredients of the mix are present in nearly all deodorants [28], popular prestige perfumes [10], perfumes used in the formulation of other cosmetic products [11], and natural-ingredient–based cosmetics [29], often in levels high enough to cause allergic reactions [30,31]. Thus, fragrance allergens are ubiquitous and virtually impossible to avoid if perfumed cosmetics are used.

CONCLUSIONS

Contact allergy to fragrance materials is common in both eczema patients and in the general population. Allergic contact dermatitis caused by perfumes and scented cosmetics is usually located in the face (including the eyelids), on the hands, and in the axillae. Patients appear to become sensitized to fragrances especially by the use of deodorant sprays and/or perfumes, and to a lesser degree by cleansing agents, deodorant sticks, or hand lotions. Thereafter, new rashes may appear or be worsened by contact with other fragranced products: cosmetics, toiletries, oral-hygiene products, household products, industrial contacts, paper and paper products, laundered fabrics and clothes, topical drugs, and fragrances used as flavors in foods and drinks.

Over 100 fragrances have been identified as allergens. The diagnosis of fragrance allergy is established by positive patch-test reactions to the fragrance mix (a mixture of eight commonly used fragrances) and/or to the patients' personal perfumes or scented products. Most reactions to the mix are relevant, i.e., fragrance allergy is the cause of the

patient's current or previous complaints, and most fragrance-sensitive patients are aware that the use of scented products may cause skin problems. One or more of the ingredients of the mix are present in nearly all deodorants, perfumes, and scented cosmetics, often in levels high enough to cause allergic reactions. Industry is advised to pay special attention to the safety evaluation of fragrance materials, notably those used in perfumes and deodorants.

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11

In Vitro Tests for Skin Irritation

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INTRODUCTION

The manufacture, transport, and marketing of chemicals and finished products requires the prior toxicological evaluation and assessment of skin corrosivity and skin irritation that might result from intended or accidental skin exposure. Traditionally, animal testing procedures have provided the data needed to assess the more severe forms of skin toxicity, an assessment requiring extrapolation of the data from the animal species to humans [1]. Current regulations may require animal test data before permission is granted for the manufacture, transport, or marketing of chemicals [2], as well as for the formulations that contain them [3].

In recent years, animal testing for dermatotoxic effects has come under increasing scrutiny and criticism from animal-rights activists for being inhumane and unnecessary. Legislation is pending that would restrict the marketing of products containing ingredients that have been tested on animals [4]. The often conflicting needs to protect worker and consumer safety, comply with regulatory statutes, and reduce animal testing procedures has led to a significant effort within industry, government, and academia to develop alternative testing methods for assessing the skin corrosion and irritation hazard of chemicals and product formulations without reliance on animal test procedures [5].

A recent example for which regulatory requirements have been coupled to the pressing need for alternative methods development is in the evaluation of skin corrosion. United States and international regulations require that chemicals be properly classified, labeled, packaged, and transported on the basis of their potential to damage or destroy tissue, including the speed with which such tissue-destructive reactions occur [2,6]. The most common animal testing methods used over the years for the evaluation of chemical corrosion potential are all based on the original method by Draize [7]. We, as well as other laboratories, have been active in the development of alternative procedures for skin-corrosion testing [8–11]. Recently, several test methods have been evaluated in an international validation program [12]. Certain of these methods should provide short-term and cost-effective alternatives to the Draize procedure, at the same time providing experimental systems for developing a better mechanistic understanding of the process of skin corrosion [8].

Skin irritation, by definition, is a less severe response than corrosion, but can span a range of responses from near corrosive at one extreme to weak cumulative or neurosensory responses at the other. The development of alternatives for skin irritation testing has lagged behind that of skin corrosion testing, likely because of the greater urgency of developing alternatives for the more severe skin responses and because of the range of responses encompassed within the ''skin irritation'' umbrella. Currently, the irritation hazard potential of chemicals is often determined through use of the same Draize procedure used for corrosion testing, the difference being mainly in the length of chemical exposure, with results used to determine labeling requirements for chemicals and products according to European Commission (EC) directives [2,3]. For noncorrosive chemicals, there has been a recent effort to develop and promote the use of clinical patch testing methods for a more relevant assessment of chemical skin irritation potential than that provided by the rabbit test [13–16]. This approach has not yet been extended to the testing of product formulations, although the European Cosmetic, Toiletry and Perfumery Association (COLIPA) has recently issued guidelines for skin-compatibility testing of cosmetic formulations in man [17]. The major problem of human testing for skin irritation or compatibility is the extended duration and relatively high cost of this clinical testing. In vitro skin irritation test methods could be used to rank chemicals or formulations for skin irritation potential, even at the low end of the irritation spectrum [18,19]. These methods (and others under development elsewhere) might provide for short-term, cost-effective approaches for screening chemicals and product formulations of interest, so that only those with satisfactory skin irritation profiles would undergo longer and more costly clinical evaluations.

This chapter will provide a brief summary of the developmental status of in vitro skin irritation test methods. It includes a brief description and update on the current validation status of skin corrosion tests. Then, it summarizes ongoing efforts in our laboratory, and the work of others, towards development of a battery of skin irritation tests that might predict varying degrees of skin irritation potential of chemicals and formulations, including many with relatively mild clinical skin irritation properties.

SKIN CORROSION TESTING

Assay Systems

Screening of chemicals for skin corrosion properties in vitro has followed three general formats. These include 1) changes in electrical conductance across intact skin (rat or human), 2) breaching of noncellular biobarriers, and 3) cellular cytotoxicity in skin or epidermal equivalent cell culture systems. Each of these systems has been subject to intra- and interlaboratory development, evaluation, and validation.

Skin corrosivity has been distinguished from skin irritation in two important ways. First, corrosive skin reactions generally occur soon after chemical exposure and are irreversible. Second, it is thought that the major processes leading to chemical corrosivity are more commonly physicochemical in nature rather than the result of inflammatory biological events [11], although inflammation is a common consequence of skin corrosion.

Initial efforts to develop a screening test for skin corrosivity examined the effects of chemical exposure on barrier function of skin through assessment of changes in the resistance of the exposed skin to transmission of electric current [20]. This test method, called transcutaneous electrical resistance (TER), was based on early studies of the electri-

cal resistance properties of skin [21] and has been developed as a corrosivity assay over the past 15 years using either rat or human skin [9,11,20,22–26]. In the TER assay, full-thickness skin is stretched over a hollow tube opening with the stratum corneum side exposed to the lumen. Test materials are applied to the skin surface for varying periods of time while the skin is immersed in buffer. After chemical exposure, the electrical resistance of the skin is measured. TER values empirically established as corrosion thresholds have been set at 4 K ohms for rat skin and 11 K ohms for human skin [9,11]. The current validation status of this assay is described in the following section.

The biobarrier destruction assay approach for corrosivity testing is exemplified by the commercial Corrositex® assay system manufactured by In Vitro International (Irvine, CA). Like the TER assay, the premise here is physicochemical destruction of a barrier by direct chemical action of a test material. Instead of intact stratum corneum, the Corrositex® assay relies on a macromolecular protein matrix as the barrier. Chemicals that breach this barrier come into contact with an underlying chemical detection system (CDS). A color change indicates penetration of the test material into the CDS. The speed with which the color change occurs after application of the chemical to the biobarrier is proportional to the severity of corrosive action. A summary of results on 75 chemicals and detergent-based formulations has been published [10], as well as a recent study on the corrosivity of organosilicon compounds [27]. An update of the current validation status of this assay is provided in the following section.

A variety of cell-based biological assay systems have been developed over the past 10 years to investigate the dermatotoxic effects of chemicals and product formulations on the skin. These have included simple submerged cell cultures, submerged cell cocultures incorporating more than a single cell type, and, more recently, the development of full-thickness skin and epidermal equivalent systems. The latter are characterized by stratified epidermal cell layers and a multilayered stratum corneum. The full-thickness culture systems also have different types of cellular and macromolecular matrices serving as a dermal element. These systems have undergone extensive development and evaluation in various academic and commercial laboratories [28–38]. We have recently reviewed features of many of the submerged and skin/epidermal equivalent cell systems [39,40]. A few of these systems have been used to develop skin corrosion screening assays [8,27]. A review of the current validation status of those assays is presented in the following section.

Validation Status

In the early 1990s a program was initiated under the auspices of the European Center for the Validation of Alternative Methods (ECVAM) to develop and validate alternative methods for the assessment of skin corrosion. This program focused on three assay systems, the TER, Corrositex®, and Skin^{2}® systems. The Skin^{2} system was a commercial ‘‘skin equivalent’’ culture system, manufactured by Advanced Tissue Sciences (La Jolla, CA) and comprising human neonatal foreskin–derived dermal fibroblasts in a collagen matrix grown on nylon mesh and seeded with human neonatal foreskin–derived epidermal keratinocytes to form a stratified and cornified epidermal component. A prevalidation study was completed with these three assay systems in seven different laboratories to assess intralaboratory and interlaboratory consistency as well as overall sensitivity and specificity of the assays in identifying known corrosive and noncorrosive chemicals. The results of the prevalidation study were published in 1995 [41]. All three tests performed well, and

no firm conclusions could be drawn as to the superiority or inferiority of one test versus the others. Individual tests had specific problems that warranted further study. These problems included relatively low specificity (TER), a high number of incompatible chemicals (Corrositex), and an inferior interlaboratory consistency profile (Skin^2). It was recommended that effort be made to address these individual deficiencies and that each assay be further evaluated in a future validation study.

The formal ECVAM-sponsored skin corrosivity validation study began in early 1995 and was completed in October 1997 with the submission of the study findings [12]. In addition to the assays included in the prevalidation work (TER, Corrositex, and Skin^2), the validation study included a second commercially available skin equivalent culture construct, Episkin® (Chaponost, France). Each assay was evaluated by three independent test laboratories, and each laboratory evaluated only one of the four assays. Hence, 12 laboratories participated in the validation study. A total of 60 corrosive and noncorrosive chemicals from a variety of chemical classes (including organic and inorganic acids and bases, neutral organics, phenols, inorganic salts, electrophiles, and soaps/surfactants) were tested [42].

All four assay systems showed acceptable intralaboratory and interlaboratory reproducibility, and all but Corrositex were applicable to the testing of all the selected chemicals. Two of the assays, TER and Episkin met the first of two major objectives of the validation study. They were capable of distinguishing corrosive from noncorrosive chemicals with acceptable rates of under- or overprediction. Only the Episkin assay system met the second major objective of the study, the ability to distinguish between known R35 (United Nations packing group I) and R34 (UN packing group II/III) chemicals across all of the chemical classes. Only 60% of the test chemicals could be adequately evaluated by the Corrositex assay. For this reason, it did not meet the criteria for a validated replacement test, although it might be valid for certain chemical classes. The Skin^2 assay system showed high specificity (100% of noncorrosive chemicals were properly identified) but low sensitivity (only 43% of corrosive chemicals were correctly identified). It also performed poorly with respect to distinguishing known R35 and R34 chemicals. Only 35% of the assays conducted on these chemicals resulted in proper classification. Previously, both the Skin^2 and Corrositex assays had received exemptions from the U.S. Department of Transportation as valid alternatives to assess skin corrosivity based on more limited evaluation. It is not certain what effect the recent ECVAM-sponsored study will have on the exemption status of these assays, although for the Skin^2 assay it is a moot point given that this culture system is no longer commercially available.

SKIN IRRITATION TESTING

Our Experience

Introduction

As previously indicated, development of in vitro methods to assess skin irritation is complicated by the fact that skin irritation encompasses a range of clinical responses from near corrosive at one extreme to very mild (perhaps sensory only) skin responses at the other. Hence, we believe that test methods and prediction models will need to be optimized for different categories of test materials or formulations and for anticipated ranges of irritation severity. That is the approach we have taken in developing in vitro skin irritation test methods for several chemical and product categories [32,39,40].

Methods

Cell Cultures. The culture system used in our studies was a stratified epidermal culture with a stratum corneum obtained from MatTek Corp. (EpiDerm® No. EPI-100; Ashland, MA). These cultures were composed of a multilayered and differentiated epidermis and multilayered stratum corneum seeded onto a permeable transwell filter. On arrival, the cultures were placed at 4°C until used for experiments (within 24 h). Before treatment, the cultures were aseptically transferred to 6-well culture plates containing assay medium.

Treatments. Test materials were reagent grade chemicals from Sigma Chemical Co. (St. Louis, MO), Aldrich Chemical Co. (Milwaukee, WI), or The Procter & Gamble Co. (Cincinnati, OH). Test-product formulations were obtained from The Procter & Gamble Co. Application of test materials to skin-equivalent cultures was as previously described [32].

MTT Viability Assay. The MTT assay is a colorimetric method of determining cell viability based on reduction of the yellow tetrazolium salt 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide (Sigma Chemical Co., St. Louis, MO) to a purple formazan dye by mitochondrial succinate dehydrogenase in viable cells [43]. This assay was performed as previously described [8].

Enzyme-Release Assay. At the end of the test material and control treatment exposures, the assay medium from under each treated or control skin culture was collected in plastic vials and immediately analyzed for lactate dehydrogenase (LDH) and aspartate-aminotransferase (AST) enzymes. The enzymes, LDH and AST, were analyzed using a colorimetric method performed with a Hitachi 717 autoanalyser with commercial test kits (Boehringer Mannheim Corp., Indianapolis, IN).

Interleukin-1α Assay. Assay medium was recovered from treated and control skin cultures (EPI-100) and stored at −20°C until analyzed. Interleukin-1α (IL-1α) was assayed with a specific enzyme-linked immunoassay kit (Quantikine; R&D Systems, Inc., Minneapolis, MN).

Results

In vitro methods for screening product formulations for mild to moderate irritation potential can aid selection of formulations for further clinical evaluation. Our approach has been to directly compare in vitro assay endpoints to in vivo human skin responses using historic or concurrent skin-response data for products and ingredients including surfactants, cosmetics, antiperspirants, and deodorants. For the in vitro studies we evaluated the cornified human epidermal skin cultures (EpiDerm, MatTek, EPI-100) dosing neat or diluted test substances to the stratum corneum surface of the skin cultures. The in vitro endpoints included the MTT metabolism assay of cell viability, enzyme release (lactate dehydrogenase and aspartate aminotransferase), and inflammatory cytokine (IL-1α) release.

We have been able to rank order chemicals (surfactants), product formulations and control materials in the in vitro and clinical studies to determine the value of the EpiDerm assay system in providing a clinically relevant ordering of irritancy potential. Whereas

the details of these results are presented elsewhere [19], Table 1 provides a summary of results to date. The in vitro rank ordering has been highly predictive of both surfactant and formulation irritancy. Surfactants (anionic, nonionic, and amphoteric) were tested in vivo using three repeat 24-hour exposures under occluded patch, and cumulative erythema grades were determined for each material. The in vitro irritancy was assessed using the MTT cytotoxicity assay. With the exception of one nonionic surfactant, the rank ordering of irritation was the same for the in vivo and in vitro tests. For antiperspirants/deodorants, the clinical irritation data were derived from home-use study diaries. The in vitro data included MTT, enzyme-release, and IL-1α assays. All showed good correlation with the human data, but the IL-1α assay showed the greatest correlation along the entire range of irritation. For cosmetics, the clinical data were derived from cumulative irritation tests where benchmark materials (0.05% and 0.1% sodium lauryl sulfate [SLS]) were included as high-irritant controls. The cumulative irritation indices for different cosmetic formula-

TABLE 1 Rank Ordering of Irritation Within Chemical or Product Classes[a]

Material/product class	Test substance	Potency rank order	
		In vivo[b]	In vitro[c]
Surfactants	.01% SLS	1	1
	.02% AE[d]/A	2	3
	.02% AE/B	3	4
	.02% AE/C	4	5
	0.6% Nonionic A	5	6
	0.2% Amphoteric	6	7
	0.6% Nonionic B	7	2
Antiperspirants/ deodorants	GD-2F[e]	1	1
	GD-2M	1	2
	GSOC	3	3
	GDF	3	5
	GSO	5	6
	HER	6	4
	HEU	7	6
Cosmetics/controls	0.1% SLS	1	1
	COS-4[f]	2	2
	0.05% SLS	3	3
	COS-3	4	4
	COS-2	5	6
	COS-1	6	5

[a] Irritation rank ordering: 1 = most irritating or cytotoxic, 7 = least irritating or cytotoxic.
[b] In vivo data were obtained from three repeat 24-hour exposure patch tests (surfactants), from home-use study diaries (antiperspirants/deodorants), or from cumulative irritation patch tests (cosmetics).
[c] Surfactants were tested in vitro by the MTT assay and antiperspirants/deodorants and cosmetics were tested by the IL-1α assay.
[d] Alkyl ethoxylate.
[e] Product codes (antiperspirants/deodorants); tested in vitro as is.
[f] Product codes (cosmetics); tested in vitro as is.
Source: Ref. 39.

tions were compared with the in vitro test data. Again, the IL-1α assays provide the best correlation with the human data across the entire range of clinical irritation responses.

Other Literature

A number of other laboratories have used various constructs of skin cultures to examine the in vitro irritation potential of chemicals and formulations. The developers of the EpiDerm cultures examined dose-response profiles to surfactants and surfactant-containing formulations, and found a good correlation between residual cell viability measures and clinical irritation profiles [44]. Later testing of chemical irritants and allergens showed a comparable irritant response profile regardless of whether cytotoxicity or cytokine release was measured. However, cytokine release in response to contact allergens occurred at noncytotoxic doses and was thought to provide additional mechanistic and perhaps a predictive application for these cultures [45]. Recently, the EpiDerm system has been used by a group from Unilever (Sharnbrook, U.K.) to examine the cytotoxicity patterns of mixed surfactants [46]. They found that, in vitro as in vivo, mixtures of surfactants produce less irritation than expected based on the irritation properties of the individual components of the mixture, a phenomenon known as antagonism.

A group from Leiden University (Leiden, The Netherlands) has been developing and applying their own unique skin-culture system to the assessment of skin irritation responses. They have used a system comprising epidermal keratinocytes seeded on de-epidermized dermis (RE-DED) and have tested various skin irritants [34,36]. This group confirmed the ability of the RE-DED system to effectively assess skin irritation potential of the anionic surfactant sodium lauryl sulfate [36]. They also showed that in vitro skin irritation patterns for oleic acid were different in submerged keratinocyte cultures versus the RE-DED system [34]. In the latter, higher doses were required because of the requirement for the chemical to penetrate the barrier. Of course, the irritation potential of acids and bases can also be underestimated in submerged cultures because of the buffering effects of the culture media [32,39,40].

Quite recently, another group of researchers (Lyon, France) have used skin-equivalent culture systems to examine the irritation potential of cosmetic product formulations. Testing cosmetic formulations of various types (creams, lotions, oils, mascaras), they observed a good correlation between in vitro indices of irritation and previously known Draize irritation indices [47,48]. Like our group, they have used viability, enzyme release, and IL-1α release to profile in vitro skin irritation. All of the above results point to the utility of skin-equivalent culture systems to detect skin irritation responses in vitro in a manner consistent with the clinical skin irritation properties of the chemicals. They offer opportunities for the further development of valid alternative test methods.

DISCUSSION

It has been important to validate the relevance of in vitro skin irritation endpoints to in vivo toxicity by confirming the presence of these endpoints in skin models representing various levels of skin organization, from intact skin to isolated cell cultures. The initial response of human cells to chemical irritants is cell damage, ranging from subtle perturbations or biochemical changes to cell death. As a response to damage, skin cells release inflammatory mediators and cytokines to initiate a local inflammation response, resulting

in the visual hallmark of erythema and edema attributable to increased blood flow and leakage of plasma from blood vessels [32,49,50].

The isolated keratinocyte culture represents the simplest of the test systems for evaluating skin irritancy in vitro. For test materials compatible with the aqueous culture medium, there has been an excellent correlation shown between human irritation potential and in vitro cytotoxicity over several orders of magnitude [32]. However, many types of chemicals (particularly acids, alkalis, and oxidants) are incompatible with the assay system. For acids and alkalis, the buffering capacity of the medium will interfere with their evaluation if pH is a key factor in their in vivo irritancy. Formulations are also difficult to test in vitro because, from a pharmacokinetic standpoint, conditions of exposure of viable keratinocytes to key irritant components of the formulation may be quite different in the culture system versus intact skin. Lastly, skin irritation can sometimes be overpredicted in these submerged cultures because they bypass the need for chemicals to penetrate a stratum corneum barrier [34].

In the late 1980s, cultured human-skin models were developed to provide a hopeful therapeutic approach to skin transplantation. An offshoot of this technology was to provide skin-equivalent culture systems for dermatotoxicity testing. Although clearly not the same as intact skin, these cultures provided a three-dimensional model of skin with the major structural components intact. The availability of cornified versions of these culture systems has provided for a major advance in development and validation of in vitro skin corrosion and irritation test methods. Although still lacking key cellular elements, these culture systems have very similar structural features as intact skin, including many of the same structural proteins, although they are generally more permeable than intact skin. The major advantage of these cultures is the ability to test anything that can be applied to and tested on intact human skin, including highly toxic materials. Validation testing has verified the ability of at least certain constructs to predict the corrosive potential of chemicals of different classes [12].

Use of these cultures for testing milder materials (e.g., cosmetics) provides a tool for early screening of new product formulations in a time- and cost-effective manner prior to more costly clinical evaluations. They also provide a means to investigate mechanisms of skin irritation. Our early efforts using cornified culture systems to screen and rank order the mild to moderate skin irritation potential of product ingredients and formulations have been highly successful [18,19]. It is well known that the irritation potential of any material in vivo is a function of both concentration and time of exposure. The in vitro testing of materials that are relatively mild after acute testing, and produce clinical irritation only after chronic or repeated exposure, is complicated by the limited duration of exposure possible in vitro. In the development of more sensitive in vitro methods, we are looking to extend the duration of exposure as much as the cultures will allow and/or use noncornified culture systems. Clearly, any increase in permeability of the culture systems versus intact skin (often viewed as a negative property for many applications) can be a benefit for the skin irritation assessment of relatively mild chemicals or product formulations. In addition, skin irritation responses in epidermal skin equivalents, with and without dermal components, are being investigated.

Although the development of one skin-equivalent culture system and the TER assay have achieved validation status under the recent ECVAM recommendation, the same is not true for skin irritation assessment. An ECVAM task force recently summarized the status of alternative methods for skin irritation testing [51]. A major recommendation was to continue development of reconstituted human-skin models and preliminary prediction

models for their use in predictive skin irritation testing. In addition, it was noted that ethical human-skin testing procedures are being developed for skin irritation hazard assessment [13–16,52] and deserve consideration in the hierarchical scheme of skin irritation testing [51].

Many issues remain unanswered in the future development of cell-based in vitro assays for skin toxicity. Continued interlaboratory validation is needed to enhance acceptance into the regulatory evaluation and approval process. Further refinement and development of irritation testing methods will enhance the utility of the models for screening purposes. Included is the development of ''flanker'' models that contain additional epidermal cell types such as melanocytes or Langerhans cells. For example, MatTek (Ashland, MA) has developed a melanocyte containing epidermal model (MelanoDerm®) and is investigating its use in UVB-protection studies [53]. Finally, the increased reliance on these models for toxicity testing and irritation screening has also created concerns over their long-term commercial supply. Increased use of high-quality culture systems and continued efforts to validate methods using these cultures may help in this process and thus ensure future access to this important technology.

NOTE ADDED IN PROOF

In the months since the submission of this chapter, several advances have occurred in the field of in vitro skin corrosion and irritation testing. In addition to the TER and Episkin assays, a second skin construct, EpiDerm, has now completed successful 'catch-up' validation [54,55] and has been endorsed by ECVAM as an alternative skin corrosivity test [56]. Also, the noncellular corrosion assay, Corrositex, was cited by the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as equivalent to the Draize test for predicting corrosivity and noncorrosivity for specified chemical classes (acids and bases) [57]. In the European Union, a new test method on skin corrosion (including the rat skin TER and human skin model assays) has just been incorporated into Annex V of Directive 67/548/EEC [58], and a draft guideline on in vitro tests for skin corrosion is under consideration by the Organization for Economic Cooperation and Development (OECD) member countries. In regard to in vitro skin irritation test methods, efforts are currently underway to identify potential in vitro acute skin irritation test methods and evaluate them through rigorous prevalidation and validation studies [59].

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In Vivo Irritation

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INTRODUCTION

Irritant Dermatitis

Skin irritation is a localized nonimmunologically mediated inflammatory process. It may manifest objectively with skin changes such as erythema, edema, and vesiculation, or subjectively with the complaints of burning, stinging, or itching, with no detectable visible or microscopic changes. Several forms of objective irritation exist (see Table 1). Acute irritant dermatitis may follow a single, usually accidental, exposure to a potent irritant and generally heals soon after exposure. An irritant reaction may be seen in individuals such as hairdressers and wet-work performing employees, who are more extensively and regularly exposed to irritants. Repeated irritant reactions may develop into a contact dermatitis, which generally has a good prognosis. Other forms of irritant dermatitis include delayed acute irritant contact dermatitis, which occurs when there is a delay between exposure and inflammation, and cumulative irritant dermatitis, which is the most common form of irritant contact dermatitis. After exposure, an acute irritant dermatitis is not seen but invisible skin changes occur, which eventually lead to an irritant dermatitis when exposure reaches a threshold point. This may follow days, weeks, or years of exposure [1]. These various forms require specialized models to predict their occurrence after exposure to specific products.

Need for Models

Prevention of skin irritation is important for both the consumer who will suffer from it and for the industry, which needs a licensable and marketable product. Accurate prediction of the irritation potential of industrial, pharmaceutical, and cosmetic materials is therefore necessary for the consumer health and safety and for product development. Presently, animal models fulfill licensing criteria for regulatory bodies. In the European Union, animal testing for cosmetics was to be banned in 1998; however, the deadline was extended to June 30, 2000 because scientifically validated models were not available. Until alternative models can be substituted, in vivo models provide a means by which a cosmetic can be

Table 1 Classification of Irritant Dermatitis

Classification	Features	Clinical picture
Acute irritant dermatitis	Single exposure Strong irritant Individual predisposition considered generally unimportant	Reaction usually restricted to exposed area, appears within minutes Erythema, edema, blisters, bullae, pustules, later eschar formation Symptoms include burning, stinging, and pain Possible secondary infection Good prognosis
Irritant reaction	Follows repeated acute skin irritation Often occupational; hairdressers, wet workers	Repeated irritant reactions may develop into contact dermatitis Good prognosis
Cumulative irritant dermatitis	Repeated exposure required Initial exposures cause invisible damage Exposure may be weeks, months, or years until dermatitis develops Individual variation is seen	Initially subject may experience stinging or burning Eventually erythema, edema, or scaling appears Variable prognosis
Delayed acute irritant contact dermatitis	Latent period of 12–24 hours between exposure and dermatitis	Clinically similar to acute irritant dermatitis Good prognosis
Subclinical irritation	Irritation detectable by bioengineering methods prior to development of irritant dermatitis	
Subjective irritation	Subject complains of irritant symptoms with no clinically visible irritation	Perceived burning, stinging, or itching
Traumatic irritant dermatitis	Follows acute skin trauma, e.g., burn or laceration	Incomplete healing, followed by erythema, vesicles, vesicopapules, and scaling; may later resemble nummular (coin-shaped) dermatitis.
Pustular and acneiform dermatitis	Caused by metals, oils, greases, tar, asphalt, chlorinated napthalenes, polyhalogenated naphthalenes, cosmetics	Develops over weeks to months Variable prognosis
Friction dermatitis	Caused by friction trauma	Sometimes seen on hands and knees

tested on living skin, at various sites, and under conditions that should closely mimic the intended human use.

Many aspects of irritation have been described, ranging from the visible erythema and edema to molecular mediators such as interleukins and prostaglandins. Therefore, a variety of in vivo and in vitro approaches to experimental assay are possible. However, no model assays inflammation in its entirety. Each model is limited by our ability to interpret and extrapolate of the features of inflammation to the desired context. Therefore, predicting human responses based on data from nonhuman models requires particular care.

Various human experimental models have been proposed, providing irritant data for the relevant species. Human models allow the substance to be tested in the manner that the general public will use it; e.g., wash testing (see the following section) attempts to mimic the consumer's use of soaps and other surfactants. Also, humans are able to provide subjective data on the degree of irritation caused by the product. However, human studies are also limited by pitfalls in interpretation, and by the fear of applying new substances to human skin before their irritant potential has been evaluated.

ANIMAL MODELS

Draize Rabbit Models

The Draize model [2] and its modifications are commonly used to assay skin irritation using albino rabbits. Various governmental agencies have adopted these methods as standard test procedure. The procedure adopted in the U.S. Federal Hazardous Substance Act (FHSA) is described in Tables 2 and 3 [3,4,5]. Table 4 compares this method some other modifications of the Draize model.

Draize used this scoring system to calculate the primary irritation index (PII). This is calculated by averaging the erythema scores and the edema scores of all sites (abraded and nonabraded). These two averages are then added together to give the PII value. A value of less than 2 was considered nonirritating, 2 to 5 mildly irritating, and greater than 5 severely irritating. A value of 5 defines an irritant by Consumer Product Safety Commission (CPSC) standards. Subsequent laboratory and clinical experience has shown the value judgments (i.e., non-, mild, and severely irritating) proposed in 1944 requires clinical judgment and perspective, and should not be viewed in an absolute sense. Many materials irritating to the rabbit may be well tolerated by human skin.

TABLE 2 Draize-FHSA Model

Number of animals	6 albino rabbits (clipped)
Test sites	2×1 inch^2 sites on dorsum
	One site intact, the other abraded, e.g., with hypodermic needle
Test materials	Applied undiluted to both test sites
	Liquids: 0.5 mL
	Solids/semisolids: 0.5g
Occlusion	1 inch^2 surgical gauze over each test site
	Rubberized cloth over entire trunk
Occlusion period	24 hours
Assessment	24 and 72 hours
	Visual scoring system

TABLE 3 Draize-FHSA Scoring System

	Score
Erythema and eschar formation	0
No erythema	
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised >1 mm)	3
Severe edema (raised >1 mm and extending beyond the area of exposure)	4

Source: Ref. 4.

Although the Draize scoring system does not include vesiculation, ulceration, and severe eschar formation, all of the Draize-type tests are used to evaluate corrosion as well as irritation. When severe and potentially irreversible reactions occur, the test sites are further observed on days 7 and 14, or later if necessary.

Modifications to the Draize assay have attempted to improve its prediction of human experience. The model is criticized for inadequately differentiating between mild and moderate irritants. However, it serves well in hazard identification, often overpredicting the severity of human skin reactions [5]. Therefore, Draize assays continue to be recommended by regulatory bodies for drugs and industrial chemicals.

Cumulative Irritation Assays

Several assays study the effects of cumulative exposure to a potential irritant. Justice et al. [6] administered seven applications of surfactant solutions at 10-minute intervals to the clipped dorsum of albino mice. The test site was occluded with a rubber dam to prevent evaporation and the skin was examined microscopically for epidermal erosion.

Frosch et al. [7] described the guinea pig repeat irritation test (RIT) to evaluate protective creams against the chemical irritants sodium lauryl sulfate (SLS), sodium hydroxide (NaOH), and toluene. The irritants were applied daily for 2 weeks to shaved back skin of young guinea pigs. Barrier creams were applied to the test animals 2 hours before and immediately after exposure to the irritant. Control animals were treated with the irritant only. Erythema was measured visually, and by bioengineering methods: laser doppler flowmetry and transepidermal water loss. One barrier cream was effective against SLS and toluene, whereas the other tested was not. In a follow-up study, another allegedly protective cream failed to inhibit irritation caused by SLS and toluene and exaggerated irritation to NaOH, contrary to its recommended use [8]. The RIT is proposed as an animal model to test the efficacy of barrier creams, and a human version, described below, has also been proposed.

TABLE 4 Examples of Modified Draize Irritation Method

	Draize	FHSA	DOT	FIFRA	OECD
Number of animals	3	6	6	6	6
Abrasion/intact	Both	Both	Intact	2 of each	Intact
Dose liquids	0.5 mL undiluted	0.5 mL undiluted	0.5 mL	0.5 mL undiluted	0.5 mL
Dose solids in solvent	0.5 g	0.5 g moistened	0.5 g moistened	0.5 g	0.5 g
Exposure period (h)	24	24	4	4	4
Examination (h)	24, 72	24, 72	4, 48	0.5, 1, 24, 48, 72	0.5, 1, 24, 48, 72
Removal of test materials	Not specified	Not specified	Skin washed	Skin wiped	Skin washed
Excluded from testing	—	—	—	Toxic materials pH ≤ 2 or ≥ 11.5	Toxic materials pH ≤ 2 or ≥ 11.5

Abbreviations: FHSA, Federal Hazardous Substance Act; DOT, Department of Transportation; FIFRA, Federal Insecticide, Fungicide and Rodenticide Act; OECD, Organization for Economic Cooperation and Development.
Source: Ref. 4.

Repeat application patch tests have been developed to rank the irritant potential of products. Putative irritants are applied to the same site for 3 to 21 days, under occlusion. The degree of occlusion influences percutaneous penetration, which may in turn influence the sensitivity of the test. Patches used vary from Draize-type gauze dressings to metal chambers. Therefore, a reference irritant material is often included in the test to facilitate interpretation of the results. Various animal species have also been used, such as the guinea pig and the rabbit [9,10]. Wahlberg measured skinfold thickness with Harpenden calipers to assess the edema-producing capacity of chemicals in guinea pigs. This model showed clear dose-response relationships and discriminating power, except for acids and alkalis where no change in skinfold thickness was found.

Open application assays are also used for repeat irritation testing. Marzulli and Maibach [11] described a cumulative irritation assay in rabbits that uses open applications and control reference compounds. The test substances are applied 16 times over a 3-week period and the results are measured with a visual score for erythema and skin thickness measurements. These two parameters correlated highly. A significant correlation was also shown between the scores of 60 test substances in the rabbit and in man, suggesting that the rabbit assay is a powerful predictive model.

Anderson et al. [12] used an open application procedure in guinea pigs to rank weak irritants. A baseline response to SLS solution was obtained after 3 applications per day for 3 days to a 1 cm^2 test area. This baseline is used to compare other irritants, of which trichloroethane was the most irritant, similar to 2% SLS. Histology showed a mononuclear dermal inflammatory response.

Immersion Assay

The guinea pig immersion assay was developed to assess the irritant potential of aqueous surfactant–based solutions, but might be extended to other occupational settings such as aqueous cutting fluids. Restrained guinea pigs are immersed in the test solution while maintaining their head above water. The possibility of systemic absorption of a lethal dose restricts the study to products of limited toxic potential. Therefore, the test concentration is usually limited to 10%.

Ten guinea pigs are placed immersed in a 40°C solution for 4 hours daily for three days. A comparison group is immersed in a reference solution. Twenty-four hours after the final immersion, the animals' flanks are shaved and evaluated for erythema, edema, and fissures [13,14,15,16]. Gupta et al. [17] concomitantly tested the dermatotoxic effects of detergents in guinea pigs and humans, using the immersion test and the patch test, respectively. Epidermal erosion and a 40 to 60% increase in the histamine content of the guinea pig skin was found, in addition to a positive patch test reaction in seven of eight subjects.

Mouse Ear Model

Uttley and Van Abbe [18] applied undiluted shampoos to one ear of mice daily for four days, visually quantifying the degree of inflammation as vessel dilatation, erythema, and edema. Patrick and Maibach [19] measured ear thickness to quantify the inflammatory response to surfactant–based products and other chemicals. This allowed quantification of dose-response relationships and comparison of chemicals. Inoue et al. [20] used this model to compare the mechanism of mustard oil–induced skin inflammation to the mechanism of capsaicin-induced inflammation. Mice were pretreated with various receptor an-

tagonists, such as 5-HT_2, H_1, and tachykinin antagonists, showing that the tachykinin NK1 receptor was an important mediator of inflammation induced by mustard oil. The mouse models provide simplicity and objective measurements. Relevance for man requires elucidation.

Other Methods

Several other assays of skin irritation have been suggested. Humphrey [21] quantified the amount of Evans blue dye recovered from rat skin after exposure to skin irritants. Trush et al. [22] used myeloperoxidase in polymorphonuclear leukocytes as a biomarker for cutaneous inflammation.

HUMAN MODELS

Human models for skin irritation testing are species relevant, thereby eliminating the precarious extrapolation of animal and in vitro data to the human setting. As the required test area is small, several products or concentrations can be tested simultaneously and compared. Inclusion of a reference irritant substance facilitates interpretation of the irritant potential of the test substances. Prior animal or in vitro studies, depending on model relevance and regulatory issue, can be used to exclude particularly toxic substances or concentrations before human exposure.

Single-Application Patch Testing

The National Academy of Sciences (NAS) [23] outlined a single-application patch test procedure determining skin irritation in humans. Occlusive patches may be applied to the intrascapular region of the back or the volar surface of the forearms, using a relatively nonocclusive tape for new or volatile materials. More occlusive tapes or chambers generally increase the severity of the responses. A reference material is included in each battery of patches.

The exposure time may vary to suit the study. NAS suggests a 4-hour exposure period, although it may be desirable to test new or volatile materials for 30 minutes to 1 hour. Studies longer than 24 hours have been performed. Skin responses are evaluated 30 minutes to 1 hour after removal of the patch, using the animal Draize scale (Table 2) or similar. Kligman and Wooding [24] described statistical analysis on test data to calculate the IT50 (time to produce imitation in 50% of the subjects) and the ID50 (dose required to produce irritation in 50% of the subjects after a 24-hour exposure).

Robinson et al. [25] suggested a 4-hour patch test as an alternative to animal testing. Assessing erythema by visual scoring, they tested a variety of irritants on Caucasians and Asians. A relative ranking of irritancy was obtained using 20% SLS as a benchmark. Taking this model further, McFadden et al. [26] investigated the threshold of skin irritation in the six different skin types. Again using SLS as a benchmark, they defined the skin irritant threshold as the lowest concentration of SLS that would produce skin irritation under the 4-hour occluded patch conditions. They found no significant difference in irritation between the skin types.

Cumulative Irritation Testing

Lanman et al. [27] and Phillips et al. [9] described a cumulative irritation assay, which has become known as the ‘‘21-day’’ cumulative irritation assay. The purpose of the test

was to screen new formulas before marketing. A 1 inch square of Webril was saturated with liquid of 0.5 g of viscous substances and applied to the surface of the pad to be applied to the skin. The patch was applied to the upper back and sealed with occlusive tape. The patch is removed after 24 hours, and then reapplied after examination of the test site. This is repeated for 21 days and the IT50 can then be calculated. Note that the interpretation of the data is best done by comparing the data to an internal standard for which human clinical experience exists.

Modifications have been made to this method. The chamber scarification test (see the following) was developed to predict the effect of repeated applications of a potential irritant to damaged skin, rather than healthy skin. The cumulative patch test described above had failed to predict adverse reactions to skin damaged by acne or shaving, or sensitive areas such as the face [28].

Wigger-Alberti et al. [29] compared two cumulative models by testing skin reaction to metalworking fluids (MWF). Irritation was assessed by visual scoring, transepidermal water loss, and chromametry. In the first method, MWF were applied with Finn Chambers on the volunteers' midback, removed after 1 day of exposure, and reapplied for a further 2 days. In the second method, cumulative irritant contact dermatitis was induced using a repetitive irritation test for 2 weeks (omitting weekends) for 6 hours per day. The 3-day model was preferred because of its shorter duration and better discrimination of irritancy. For low-irritancy materials in which discrimination is not defined with visual and palpatory scores, bioengineering methods (i.e., transepidermal water loss) may be helpful.

The Chamber Scarification Test

This test was developed [30,31] to test the irritant potential of products on damaged skin. Six to eight 1 mm sites on the volar forearm were scratched eight times with a 30-gauge needle without causing bleeding. Four scratches were parallel and the other four are perpendicular to these. Duhring chambers, containing 0.1 g of test material (ointments, creams, or powders), were then placed over the test sites. For liquids, a fitted pad saturated (0.1 mL) may be used. Chambers containing fresh materials are reapplied daily for 3 days. the sites are evaluated by visual scoring 30 minutes after removal of the final set of chambers. A scarification index may be calculated if both normal and scarified skin are tested to reflect the relative degree of irritation between compromised and intact skin; this is the score of scarified sites divided by the score of intact sites. However, the relationship of this assay to routine use of substances on damaged skin remains to be established. Another compromised skin model, the arm immersion model of compromised skin, is described in the following immersion tests section.

The Soap Chamber Test

Frosch & Kligman [32] proposed a model to compare the potential of bar soaps to cause ''chapping.'' Standard patch testing was able to predict erythema, but unable to predict the dryness, flaking, and fissuring seen clinically. In this method, Duhring chambers fitted with Webril pads were used to apply 0.1 mL of an 8% soap solution to the human forearm. The chambers were secured with porous tape, and applied for 24 hours on day 1. On days 2 to 5, fresh patches were applied for 6 hours. The skin is examined daily before patch application and on day 8, the final study day. No patches are applied after day 5. Applica-

tions were discontinued if severe erythema was noted at any point. Reactions were scored on a visual scale of erythema, scaling, and fissures. This test correlated well with skin-washing procedures, but tended to overpredict the irritancy of some substances [33].

Immersion Tests

These tests of soaps and detergents were developed in order to improve irritancy prediction by mimicking consumer use. Kooyman & Snyder [34] describe a method in which soap solutions of up to 3% are prepared in troughs. The temperature was maintained at 105°F while subjects immersed one hand and forearm in each trough, comparing different products (or concentrations). The exposure period ranged from 10 to 15 minutes, three times each day for 5 days, or until irritation was observed in both arms. The antecubital fossa was the first site to show irritation, followed by the hands [6,34]. Therefore, antecubital wash tests (see the following) and hand immersion assays were developed [5].

Clarys et al. [35] used a 30-minute/4-day immersion protocol to investigate the effects of temperature as well as anionic character on the degree of irritation caused by detergents. The irritation was quantified by assessment of the stratum corneum barrier function (transepidermal water loss), skin redness (a* color parameter), and skin dryness (capacitance method). Although both detergents tested significantly affected the integrity of the skin, higher anionic content and temperature, respectively, increased the irritant response.

Allenby et al. [36] describe the arm immersion model of compromised skin, which is designed to test the irritant or allergic potential of substances on damaged skin. Such skin may show an increased response, which may be negligible or undetectable in normal skin. The test subject immersed one forearm in a solution of 0.5% sodium dodecyl sulfate for 10 minutes, twice daily until the degree of erythema reached 1 to 1+ on visual scale. This degree of damage corresponded to a morning's wet domestic work. Patch tests of various irritants were applied to the dorsal and volar aspects of both the pretreated and untreated forearms, and also to the back. Each irritant produced a greater degree of reaction on the compromised skin.

Wash Tests

Hannuksela and Hannuksela [37] compared the irritant effects of a detergent in use testing and patch testing. In this study of atopic and nonatopic medical students, each subject washed the outer aspect of the one forearm with liquid detergent for 1 minute, twice daily for 1 week. Concurrently, a 48-hour chamber patch test of five concentrations of the same detergent was performed on the upper back. The irritant response was quantified by bioengineering techniques: transepidermal water loss, electrical capacitance, and skin blood flow. In the wash test, atopics and nonatopics developed irritant contact dermatitis equally, whereas atopics reacted more readily to the detergent in chamber tests. The disadvantage of the chamber test is that, under occlusion, the detergent can cause stronger irritation than it would in normal use [38]. Although the wash test simulates normal use of the product being tested, its drawback is a lack of standard guidelines for performing the test. Charbonnier et al. [39] included squamometry in their analysis of a hand-washing model of subclinical irritant dermatitis with SLS solutions. Squamometry showed a significant

difference between 0.1 and 0.75% SLS solutions whereas visual, subjective, capacitance, transepidermal water loss, and chromametry methods were unable to make the distinction. Charbonnier suggests squamometry as an adjunct to the other bioengineering methods.

Frosch [33] describes an antecubital washing test to evaluate toilet soaps, using two washing procedures per day. Simple visual scoring of the reaction (erythema and edema) allows products to be compared. This comparison can be in terms of average score, or number of washes required to produce an effect.

Assessing Protective Barriers

Zhai et al. [40] proposed a model to evaluate skin protective materials. Ten subjects were exposed to the irritants SLS and ammonium hydroxide (in urea), and Rhus allergen. The occluded test sites were on each forearm, with one control site on each. The irritant response was assessed visually using a 10-point scale, which included vesiculation and maceration unlike standard Draize scales. The scores were statistically analyzed for non-parametric data. Of the barrier creams studied, paraffin wax in cetyl alcohol was found to be the most effective in preventing irritation.

Wigger-Alberti and Elsner [41] investigated the potential of petrolatum to prevent epidermal barrier disruption induced by various irritants in a repetitive irritation test. White petrolatum was applied to the backs of 20 human subjects who were exposed to SLS, NaOH, toluene, and lactic acid. Irritation was assessed by transepidermal water loss and colorimetry in addition to visual scoring. It was concluded that petrolatum was an effective barrier cream against SLS, NaOH, and lactic acid, and moderately effective against toluene.

Frosch et al. [42] adapted the guinea pig RIT previously described for use in humans. Two barrier creams were evaluated for their ability to prevent irritation to SLS. In this repetitive model, the irritant was applied to the ventral forearm, using a glass cup, for 30 minutes daily for 2 weeks. One arm of each subject was pretreated with a barrier cream. As in the animal model, erythema was assessed by visual scoring, laser doppler flow, and transepidermal water loss. Skin color was also measured by colorimetry (La* value). The barrier cream decreased skin irritation to SLS, the most differentiating parameter being transepidermal water loss and the least differentiating being colorimetry.

Bioengineering Methods in Model Development

Many of the models previously described do not use the modern bioengineering techniques available, and therefore data based on these models may be imprecise. Despite the investigations skill, subjective assessment of erythema, edema, and other visual parameters may lead to confounding by inter and intraobserver variation. Although the eye may be more sensitive than current spectroscopy and chromametric techniques, the reproducibility and increased statistical power of such data may provide greater benefit. A combination of techniques, such as transepidermal water loss, capacitance, ultrasound, laser doppler flowmetry, spectroscopy, and chromametric analysis, in addition to skilled observation may increase the precision of the test. Andersen and Maibach [43] compared various bioengineering techniques, finding that clinically indistinguishable reactions induced significantly different changes in barrier function and vascular status. An outline of many of these techniques is provided by Patil et al. [5].

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Eye Irritation Testing

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INTRODUCTION

The eye is the sensory organ that captures visible light energy and converts it into neural impulses that give rise to vision, our most important sense. Because of its external location, the eye is constantly exposed. It can be damaged by drying, natural environmental contaminants, and micro-organisms. It is also vulnerable to injury induced by a variety of traumatic insults, including chemical exposure.

Accidental eye exposure to chemicals or consumer products occurs at home and in the workplace. Therefore, developers of consumer goods and chemicals must perform ocular safety assessments in order to prevent dangerous products from reaching the market and to correctly advise consumers and workers on the safety of the materials they use [1–3].

Data from animal tests have been used to make eye safety assessments since the 1940s. These tests use the albino rabbit as the animal model and a systematic numerical scoring system for quantifying the irritation response [4]. Although the in vivo eye irritation tests provide important and useful information, they are not without faults. Thus, there is great interest in developing alternative methods that will allow toxicologists to make accurate ocular safety assessments without using animals. Accomplishing such a goal is a great challenge.

This chapter will review the state of the art in developing nonanimal methods for the Draize eye irritation test. It will describe the anatomical and physiological features of the anterior eye relevant to ocular safety testing and development of alternative assay systems. The work that has been done to develop alternative methods will be reviewed. The chapter closes with a discussion of how alternative methods may be used in the safety assessment process and the areas where additional research is needed in order to provide more reliable tests for the future.

HUMAN OCULAR ANATOMY

The eyeball is a fibrovascular spheroid globe suspended in a bony orbit by numerous ligaments and extrinsic muscles [5,6]. The globe is lightproof except for the transparent corneal surface. Only the anterior aspect of the eyeball is exposed to the environment. The rest is protected behind the eyelids and bony orbital rim.

The eyeball has three coats that are further divided into subparts. The outer coat is the transparent cornea and the gray-white sclera that provides the primary supporting framework of the globe. The middle coat is the uvea that contains the choroid, ciliary body, and iris. The inner coat is the retina, the neural photoreceptive tissue in the eye.

The majority of the nonretinal structures perform secondary functions that aid the primary photoreception process. These include focusing images on the retina (cornea and lens), regulating the amount of light entering the eye (iris), providing nutrients to ocular tissues (vasculature, aqueous humor, vitreous humor, and lachrymal or tear system), moving the eyes (extrinsic musculature), and protection (somatosensory nerves and eyelids).

Outer Coat

Cornea and Precorneal Tear Film

The cornea is the transparent anterior surface of the eye where light passes to the retina (Fig. 1). Because the cornea is the main refractive surface of the eye, it also plays a key role in focusing images on the photoreceptor surface. A clear, properly shaped cornea is therefore critical for normal vision. Its exposed location makes it particularly vulnerable to injury, and any scarring that occurs may lead to opacities or shape changes that permanently impair vision.

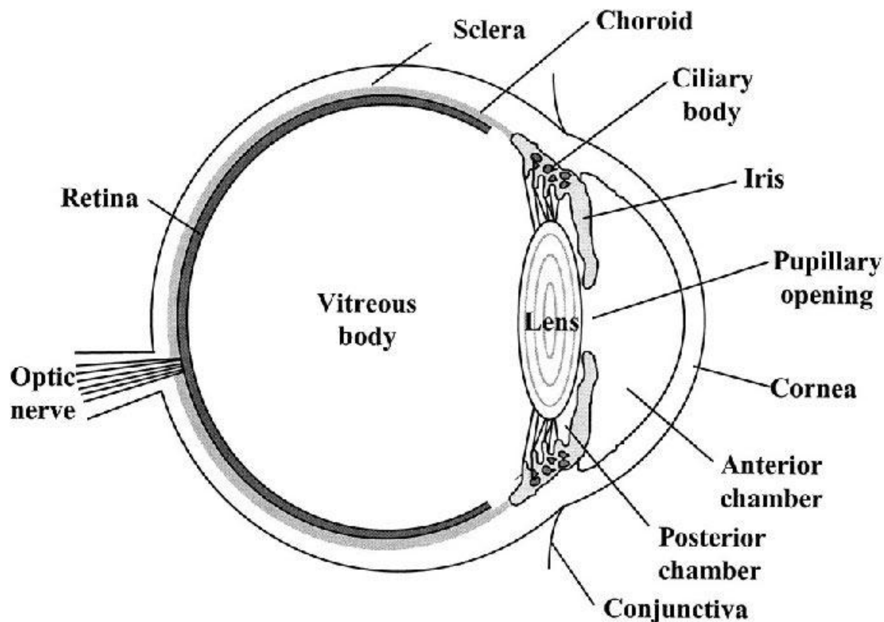

FIGURE 1 Cross section of the eye.

Precorneal Tear Film. The anterior surface of the cornea is covered by the precorneal tear film. This outer film is important for proper corneal function. It hydrates the anterior cornea and provides a smooth, continuous surface that enhances its optical properties. The tear film comprises an anterior lipid layer, with an aqueous and mucin-containing layer underneath. The lipid layer slows the evaporation of the aqueous layer, and provides a smooth, regular optical surface. The mucin wets the microvilli of the corneal epithelial cells and must be intact for the precorneal tear film to form and remain on the corneal surface.

Cornea. The cornea has three layers: the epithelium with its basement membrane, the stroma or substantia propria, and the endothelium with its basement membrane (Fig. 2).

Epithelium. In humans, the corneal epithelium is approximately 50 to 90 μm thick and covers the entire stromal surface. It is a stratified, nonkeratinized epithelium of five to six cell layers. The outermost epithelium has two to three layers of squamous cells. The midzone or wing cell layer consists of two to three layers of polyhedral cells, and the bottom-most or basal cell layer is a single layer of cells. The epithelial cells regenerate in the basal layer, and become progressively flatter as they migrate toward the surface. Epithelial stem cells reside in the basal cell layer in the more peripheral cornea (limbus), whereas transient amplifying cells lie over the cornea. The limbus is 5 to 10 cell layers thick, and overlies a rather loose and highly vascular connective tissue clearly distinct from the dense and avascular corneal stroma. It contains melanocytes and Langerhans cells, and marks the boundary of the cornea with the bulbar conjunctiva. Squamous surface

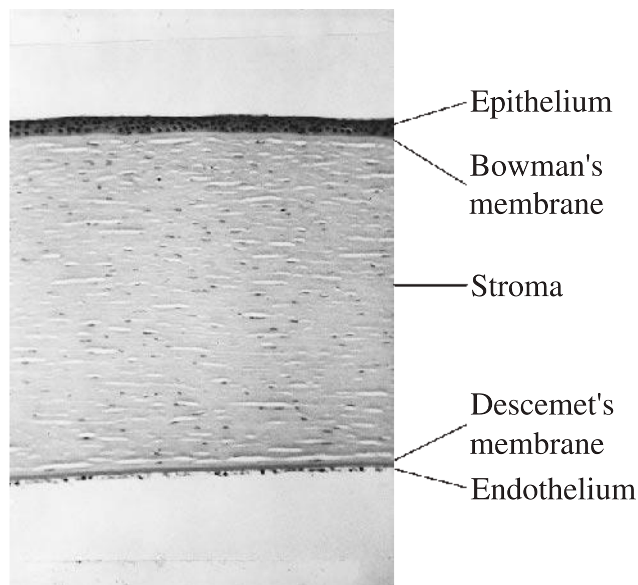

FIGURE 2 Cross section of human cornea showing from top to bottom the epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium (H&E stain, 200× magnification). (Courtesy of I. Cree, Moorefield's Eye Hospital, London, England.)

cells are shed from the surface of the cornea after approximately 7 days. Directly below the basal cell layer is the basement membrane.

Stroma. The stroma constitutes approximately 90% of the corneal thickness. Its anterior portion, Bowman's layer, is an acellular region lying just under the epithelial basement membrane. It is more resistant to deformation, trauma, passage of foreign bodies, or infecting organisms than the other layers. Once damaged, its architecture may not be restored, leading to abnormalities in corneal thickness and optical properties that could result in permanent vision deficit. The remainder of the stroma is composed of collagen fibrils gathered together in lamellae that run in parallel with the corneal surface. The fibrils within a lamella are highly organized and are surrounded by a glycosaminoglycan matrix. Corneal glycosaminoglycans are 60% keratin sulfate, and 40% chondroitin sulfates. These act as anions and bind cations and water. The posterior surface of the stroma is lined with the loosely attached Descemet's layer that is the basement membrane for the endothelial cells. Scattered throughout the lamellae are long, flat fibroblast-like cells called keratocytes. These cells have long processes that extend to adjacent cells. There are also a few neutrophils and macrophages that migrate through the stroma. Branches of the ophthalmic branch of the fifth (trigeminal) cranial nerve, which are primarily sensory, run through the anterior third of the corneal stroma and associate with the epithelium.

Endothelium. The endothelium is a single layer resting on Descemet's layer. The endothelium originates from the neural crest and therefore is not a true endothelium. The apical surface is in contact with the aqueous humor of the anterior chamber. The cells are tightly bound to each other with desmosomes. The endothelium serves the important function of maintaining the dehydration (deturgescence) that is also required to maintain corneal clarity (see the following section).

Sclera

The sclera is a dense, fibrous, collagenous structure that makes up the gray-white part of the globe. Like the cornea, it has three layers. The outermost layer is the episclera. The episclera is a vascularized connective tissue that merges with the scleral stroma and extends connective tissue bundles into the fascia surrounding the globe. The major layer of the sclera is the stroma. The stroma lies in the middle and is composed of irregularly arranged bundles of collagen fibrils. The irregular size and arrangement of these fibrils leads to the white color of the majority of the eyeball. The inner surface of the sclera is the lamina fuscia, which lies interior to the scleral stroma. It contains fine collagen fibers that form the connection between the choroid and sclera. The anterior external scleral surface of the stroma is covered by the conjunctiva. The conjunctiva is a transparent mucous membrane that covers the externally exposed scleral surface (bulbar conjunctiva) as well as the inner surface of the eyelids (palpebral conjunctiva). The conjunctival epithelium is continuous with the corneal epithelium and the lachrymal drainage system. The conjunctiva contains many blood vessels, nerves, conjunctival glands, and inflammatory cells. Small blood vessels are present throughout. They are usually not visible, but dilate and become leaky during inflammation. The nerves transmit pain responses and mediate neurogenic vasodilatation and tearing. The conjunctival glands provide moisture and secrete the constituents of the precorneal tear film.

Anterior Chamber, Posterior Chamber, and Aqueous Humor

Between the rear surface of the cornea and the front surface of the lens capsule is a fluid-filled chamber (Fig. 1). This chamber is divided into anterior and posterior regions by the

iris. These chambers are connected through the pupillary opening. The anterior chamber lies in front of the iris and the posterior chamber lies behind the iris and in front of the lens capsule.

The Middle Coat. The middle coat of the eye is the uvea. It consists of the choroid, the ciliary body, and the iris (Fig. 1). The choroid is a blood vessel–rich layer that provides blood to the retinal pigmented epithelium and outer half of the adjacent sensory retina. The ciliary body secretes the aqueous humor that fills the anterior and posterior chambers and contains the smooth muscle that alters the lens shape as needed for near and far vision. The iris is a diaphragm that lies in front of the lens and ciliary body. Contraction of iris circular or radial muscles leads to closing or opening of the pupil, respectively, which regulates the amount of light entering the eye.

The Inner Coat. The inner coat of the eye is the retina. This layer contains the neurosensory cells that transmit light-induced signals to the brain for visual interpretation. The two major parts of the retina are the inner sensory layer and the outer pigmented epithelium. The sensory layer lies between the pigmented epithelium on the outside and the vitreous humor on the inside. It is stratified into several sublayers containing the different photoreceptor and accessory cells involved with sensing and processing the light projected onto the retinal surface. The pigmented epithelium is only one layer thick and lies between the sensory epithelium and choroid. Readers interested in more details on ocular anatomy, physiology, and biochemistry should consult recent texts on the subject [7–11].

ROUTINE IN VIVO OCULAR IRRITATION TESTING

The need for ocular safety testing became clear early in the 1930s when an untested eyelash product containing p-phenylene diamine was marketed in the United States. Use of this and similar products led to sensitization of the external ocular structures, corneal ulceration, vision loss, and at least one fatality [12]. These events resulted in passage in the United States of the Food, Drug and Cosmetic Act of 1938, which required that materials sold to consumers be safe.

In response to the need for test methods to assess ocular safety, in vivo assays were developed and put into use. One of the earliest reported experimental animal procedures was devised by Friedenwald to assess the effects of acids and bases on the eye [13]. This was the first time the effects of test materials on the cornea, conjunctiva, and iris were separately recorded. Subsequently, Carpenter and Smyth [14] studied many materials and primarily recorded their effects on the cornea. Draize et al. [4] improved the test by standardizing Friedenwald's method and simplifying the scoring system. Subsequently, the Draize procedure and modifications of it have become the standard for assessing the irritancy potential of test materials for more than 50 years. The data are also used by toxicologists to assure that chemicals and consumer products (1) can be made safely in factories, (2) are safe for their intended use and any foreseeable misuse, (3) are appropriately labeled, and (4) meet regulatory safety testing requirements [15].

The Draize Eye-Irritation Test

The standard Draize eye-irritation test uses either three or six albino rabbits. Statistical studies conducted to determine the effect of reducing the number of animals used in a single study from six to three showed that a three-animal test provides eye-irritation classification similar to that obtained by using six rabbits [16,17]. Standard Draize eye-irritation

test protocols normally require that 100 μL of a test material is placed in the lower cul-de-sac of one eye, and the eyelids are held shut for a brief period of time. The untreated contralateral eye is used as the control. The eyes are sometimes rinsed after treatment to determine the effect of irrigation on the extent of irritation or to remove test substances trapped within the cul-de-sac.

Generally the eyes are examined using a pen light and graded by a technician for irritation on days 1, 2, 3, 4, and 7 after dosing and weekly thereafter. However, times at which the eyes are examined for irritation after dosing may vary because of differences in government regulations and preferences of different toxicologists. In some cases, the eyes are examined at time points earlier than day 1 (e.g., 1h, 3h). Similarly the maximum period allowed to determine recovery may vary (e.g., 3–5 weeks). Eyes are generally not examined once they have returned to normal. Examinations are sometimes augmented by fluorescein staining and slit-lamp examinations to better assess corneal changes. A grading scale has been proposed based on examinations with a slit lamp [18].

The Draize test uses a systematic numeric grading system to quantify the eye irritation response (Table 1). Changes associated with the cornea, conjunctiva, and iris are assessed by using a pen light. Scores are assigned for the various changes. The scores for the cornea, conjunctiva, and iris are weighted such that changes associated with the cornea are given the most weight, with the maximum score for the cornea being 80 out of a total possible score of 110. A test substance's potential to cause ocular irritation is then determined by assessing the individual animal scores, the maximum average score (highest mean group score during the study), and days to recovery. In general, innocuous or slightly irritating materials tend to affect only the conjunctiva, and the eye recovers in 1 to 2 days; mildly to moderately irritating materials affect the conjunctiva and cornea, and the eye recovers in days to weeks; and moderately to severely irritating materials affect the cornea, iris, and conjunctiva, and the eye recovers in weeks or not at all. These results are often further classified according to various regulatory classification schemes in use around the world. The interested reader should consult Chan and Hayes [19] for a summary of regulatory considerations.

Although the Draize eye-irritation test and slight variations of it have remained the standard procedure for determining ocular-irritation responses, the use of this test has not continued without significant criticism. The sensitivity and relevance of the Draize test have been questioned because the dose given is greater than the volume of the conjunctival cul-de-sac of the rabbit eye (30 μl) [20], thereby considerably exceeding the dose received in human accidental eye exposure [21,22]. Additionally, the in vivo tests have been criticized for their subjectivity [23], lack of repeatability [24,25], overprediction of human responses [26–28], and by animal welfare advocates because they require the use of animals [29]. Therefore, efforts have been made to develop and validate significantly modified in vivo test protocols as well as develop in vitro tests to reduce and perhaps ultimately eliminate the use of animals in ocular-irritation testing.

Modifications of the In Vivo Eye-Irritation Test

The Low-Volume Eye Test

In the early 1980s, modifications made in the amount of test material dosed and site of application resulted in a refined version of the classical Draize test, called the low-volume eye test (LVET). The LVET has been reported to be less stressful to rabbits and more predictive of human ocular irritancy potential than the standard Draize procedure

TABLE 1 Scale of Weighted Scores for Grading the Severity of Ocular Lesions

Ocular effects		Grade
Cornea		
(A)	Opacity-degree of density (area that is most dense is taken for reading)	
	Scattered or diffuse area—details of iris clearly visible	1
	Easily discernible translucent areas, details of iris clearly visible	2
	Opalescent areas, no details of iris visible, size of pupil barely discernible	3
	Opaque, iris invisible	4
(B)	Area of cornea involved	
	One quarter (or less) but not zero	1
	Greater than one quarter—less than one half	2
	Greater than one half—less than three quarters	3
	Greater than three quarters—up to whole area	4
	Total maximum* = 80	
Iris		
(A)	Values	
	Fold above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)	1
	No reaction to light, hemorrhage; gross destruction (any one or all of these)	2
	Total maximum** = 10	
Conjunctivae		
(A)	Redness (refers to palpebral conjunctivae only)	
	Vessels definitely injected above normal	1
	More diffuse, deeper crimson red, individual vessels not easily discernible	2
	Diffuse beefy red	3
(B)	Chemosis	
	Any swelling above normal (includes nictitating membrane)	1
	Obvious swelling with partial eversion of the lids	2
	Swelling with lids about half closed	3
	Swelling with lids about half closed to completely closed	4
(C)	Discharge	
	Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
	Discharge with moistening of the lids and hairs just adjacent to the lids	2
	Discharge with moistening of the lids and considerable area around the eye	3
	Total maximum† = 20	

* Score = A × B × 5.
** Score = A × 5.
† Score (A + B + C) × 2.
Note: The maximum total score is the sum of the total maximum scores obtained for the cornea, iris, and conjunctivae.
Source: Ref. 4.

[26,27,30,31]. The LVET differs from the standard Draize eye-irritation test in three ways: (1) the volume of test substance applied is 10 μL instead of 100 μL; (2) the test substance is placed directly on the corneal surface instead of into the lower conjunctival cul-de-sac; and (3) the eyes are not held shut after the test substance is applied. This method of application and the dose applied much more closely simulates accidental human exposures [32]. Normally either three or six rabbits are used per test substance. Statistical studies

similar to those conducted for the Draize test indicate that results from three rabbits provide eye-irritation classification similar to that obtained from studies using six rabbits, so that animal use in this test can be minimized [33].

Objective Measurements of Eye Injury

In addition to the LVET, other modifications have been made to the in vivo test. Most of these changes have been made in an attempt to minimize variability. Because the subjective nature of the grading is thought to be a major source of variability, work has been done to eliminate as much as possible the subjective components of the test. Some of the methods evaluated include assessing corneal thickness [34–36], water content [36,37], permeability [38–40], and surface area damaged using fluorescein, wound healing, and exfoliative cytology [41]. Objective measurements of conjunctivitis have included assessments of capillary permeability [36,37], redness, and exfoliative cytology [41]. Others have attempted to assess the utility of measuring intraocular pressure [42] and protein content of the aqueous humor [36,37]. None of these methods is in routine use.

REPLACING THE ANIMAL TEST WITH IN VITRO METHODS

Introduction

There are strong social, political, ethical, and scientific arguments for the development and use of nonanimal methods as alternatives to the Draize eye-irritation test. Alternative methods currently under investigation use a diverse set of human and animal cells, tissues, and biochemical reagents, and measure a diverse set of endpoints thought to be associated with eye-irritation responses in vivo. Few of these tests, however, attempt to model the entire eye. Instead, they usually model subparts of the larger, more complex eye-irritation response. Figure 3 shows this reductionist relationship across the spectrum of available

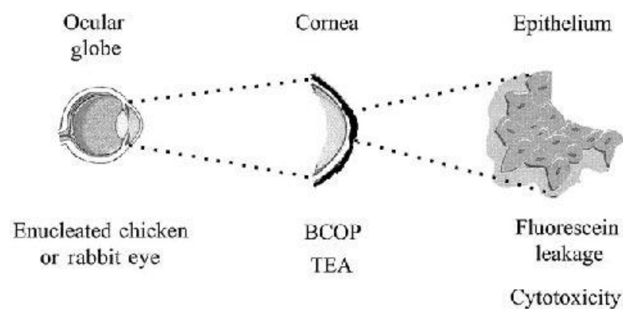

FIGURE 3 A diagram illustrating how in vitro assays have been developed to model different parts of the eye-irritation response. In the development of in vitro tests, the eye is in effect reduced to component parts. The tests developed model different parts of the eye-irritation response and allows studies on mechanisms of action. The first reduction step from the intact animal uses isolated whole eyes obtained from the abattoir. Examples include the chicken enucleated eye test and the isolated rabbit eye test. The next level of reduction is represented by tests that use isolated corneas and 3-dimensional tissue constructs. Examples include the bovine cornea opacity and permeability test (BCOP) and the topical application assays (TEA), respectively. The final level of reduction represents tests based on cell cultures containing single cell types. Examples of tests in this category include the fluorescein leakage test and other cytotoxicity tests.

in vitro methods. These methods use (1) isolated whole eyes, (2) isolated corneas, (3) multilayer (3-dimensional) single- and multicell systems, and (4) single-cell culture systems. Representatives of each of these levels will now be reviewed.

Isolated Whole Eyes

At the first stage of reduction, in vitro tests use isolated whole eyes usually obtained from an abattoir. Examples of such tests include the Isolated Rabbit Eye Test (IRE) [43–45] and the Chicken Enucleated Eye Test (CEET) [45–47]. In these model systems, test substances are applied directly to the cornea of an isolated eye for short time periods (usually around 10 sec). Subsequently, several measurements are made to estimate the severity of the resulting injury. These measurements are generally similar to those that can be made in the whole animal, including corneal opacity, corneal swelling, and fluorescein retention. Histopathological examination of the injured tissue can also be conducted. Both isolated eye models have generally performed quite well in identifying severely irritating materials; in fact, the IRE is accepted by regulatory agencies in the United Kingdom for the classification of severely irritating materials, as is the CEET in the Netherlands. Both test methods are compatible with solid and liquid test articles.

Isolated Cornea Models

The substrate used at the next level of reduction is isolated corneas (Fig. 3). The most common source of corneas for these studies is bovine eyes obtained from the abattoir. These corneas are used in an assay called the bovine cornea opacity and permeability (BCOP) test [45,48]. In this assay, test materials are applied directly to the anterior surface of corneas mounted in the center of a dual-sided organ culture chamber. After the designated exposure time, the test substance is washed away and the resulting corneal opacity and changes in epithelial barrier function, evaluated by increased permeability to fluorescein, are measured. An advantage of this model is that the corneal opacity can be measured quantitatively with a photometer because the organ chamber has transparent glass covers on each end. As with the isolated whole eye, it has been shown that assessment of histopathological changes provides additional useful information [49,50].

Multilayer (3-Dimensional) Cultures

The next level of reduction is represented by artificial 3-dimensional tissues constructed from human cells. These tissues are of two types: one is designed to model the corneal epithelium, whereas the second attempts to reconstruct the cornea in vitro.

Dermal and Corneal Epithelium Models. Because the corneal epithelium provides an important barrier function and the epithelial surface is normally the first part of the eye to contact a potentially hazardous material, several in vitro models have been developed to assess the effects of chemicals on epithelial cells. These models are generally reconstructed from human epidermal or corneal cells (either primary or immortalized cultures), which are seeded onto a specialized substrate. Under the appropriate conditions the epithelial cells stratify vertically and differentiate into 3-dimensional, nonkeratinized structures. Test material is placed directly on this substrate and injury is assessed by monitoring changes in the construct's barrier property, the release of cytokines, or cytotoxicity. For example, an immortalized human cornea cell line (10.014 pRSV-T) has been grown on cell culture inserts at an air-liquid interface so that the cultures form an epithelium containing four to six cell layers [51–53]. Test substances are applied to the epithelial surface for brief

periods (up to 5 min) in dose-response experiments. Endpoints measured include the barrier function of the epithelium using transepithelial permeability to fluorescein and electrical resistance, along with cell viability [54]. Results from this model, called HCE-T, correlate with historical rabbit-eye data for water-soluble ingredients and surfactant-based personal care products [52]. Others have reported that early (1 h) release of the cytokine interleukin 1-α is a predictive marker for surfactant responses in another human corneal epithelial cell line, CEPI 17 c1.4 [55]. Interleukin 8 appears to be a late (24 h) marker of response, although the bulk of the IL-8 response appears secondary to the release of IL-1α. Taken together, this work shows the potential utility of human cornea epithelial cells to assess effects of test substances on epithelial barrier function, viability, and inflammation, as well as to evaluate specific biochemical and molecular mechanisms of these responses.

Other models have been constructed using primary human epidermal cells rather than immortalized cell lines. Several tissues of this type are available commercially. Currently available substrates include EpiOcular [56] (MatTek Corporation, Ashland, MA) and SkinEthic cultures [57,58] (SkinEthic, Nice, France). In these assays, test substances are applied to the surface of the cultures for a specified period of time. Then, the test substance is washed away and viability of the cells is measured by using one of several vital dyes [57–61]. The release of various cytokines is also measured. These models have been shown capable of differentiating degrees of irritancy between mild test substances. Another advantage of these systems is that they have proven useful for assessing both water-soluble and water-insoluble consumer products, cosmetics, and ingredients [56,59,62].

Human Cornea Models. The development of human corneal cultures analogous to 3-dimensional human skin cultures that are used to evaluate skin irritation [63,64] is now an active area of research. Martin et al. [65] have reported on trilaminar substrates developed from early passage human corneal epithelial, stroma, and endothelial cells. Endpoints evaluated in this model include barrier function, cytotoxicity, and release of the inflammatory mediators PGE_2 and LTB_4. Development of immortalized human cornea cell lines and their incorporation into trilaminar corneal models have also been reported by Griffith and coworkers [66,67]. Functional and biochemical analysis of these cultures indicate the presence of differentiation markers and other properties similar to those found in intact human corneas. In initial characterization, cultures treated with model surfactants elicit responses similar to those observed in vivo.

Single-Cell Culture Systems, Isolated Single Cells

At the last step in the reductionist scheme are assays that use monolayer cell cultures derived from epithelial cells of eyes or other organs such as the skin. The study of interactions between test substances and single cells and monolayer cultures of various types was one of the earliest approaches evaluated for eye-irritation tests in vitro. The most commonly used endpoint is assessment of direct cytotoxicity after a short-term exposure to test articles. Examples of methods in this category include the neutral red uptake test [62,68–71], the neutral red release test [72,73], and the red blood cell lysis test [62,70,74,75]. In addition, the real-time effects of a test material on the metabolic rate of cultured cells can be assessed by using the Cytosensor microphysiometer (Molecular Devices Corp., Menlo Park, CA) [62,69,70,76–78]. The Fluorescein Leakage Test is another cytotoxicity assay that measures the capacity of a test substance to damage the barrier function normally associated with epithelial cells. With this assay, confluent monolayer

cultures of renal epithelial cells are treated with test material. After exposure the change in the capacity of the epithelial cells to block fluorescein passage is measured [62,70,79–81]. Additional information on these tests may be found in an extensive review of assays based on single-cell cultures published by U.S. Interagency Regulatory Alternatives Group (IRAG) [82,83].

Other Test Systems

There are several in vitro tests that have been evaluated extensively as alternatives for eye-irritation testing that do not fit entirely within the reductionist scheme just described. The most significant tests in this category are the chorioallantoic membrane (CAM) assays. Use of the CAM of the chicken egg as a substrate for in vitro testing was first described by Luepke et al. [84], who reasoned that the highly vascularized CAM might be an acceptable surrogate for conjunctival tissue. To this end, they developed a model called the hen's egg test–CAM (HET–CAM). In this procedure, test substances are placed directly on the CAM exposed directly underneath the air cell. The resulting hemorrhage, coagulation, and lysis appearing on the CAM are measured at defined timepoints after the test article is applied. Results from this test are accepted by regulatory agencies in Germany as adequate for identifying severe irritants. A complementary test called the CAM vascular assay (CAMVA) has also been developed [85,86]. The CAMVA differs from the HET–CAM in several ways, including the site of the egg shell that is opened (side of the egg instead of the air cell), the endpoint measured (changes in characteristics of the CAM vasculature), and the dosing scheme (serially diluted test substances instead of a single test concentration). Both the HET–CAM and the CAMVA assay are reviewed in detail in the U.S. IRAG evaluations [87]. Results from evaluation of this test in several international validation studies have been reported [62,88–90].

Practical Use of In Vitro Tests for Eye-Irritation Testing

The effort to develop and validate nonanimal test methods has significantly increased the use of these tests for assessing eye safety. Experience gained from this work has shown that the methods provide information useful for safety assessments, but the conduct and interpretation of results from in vitro tests are more complex than for standard in vivo testing. Therefore, considerable care and planning need to be undertaken before beginning a study in order to obtain reliable results. Given the increased complexity associated with in vitro testing, we have found that the use of the new methods is greatly facilitated by the establishment of a standard framework that contains four elements. These include 1) a well-defined process that specifies the steps to follow during the conduct of an eye safety assessment of a test article, 2) protocols and standard operating procedures (SOPs) that define all the tests used within the eye safety assessment process, 3) prediction models that guide the interpretation of results obtained from in vitro and other test methods, and 4) a summary document that provides practical guidance to toxicologists on how to conduct the overall process. The important aspects of each of these elements will now be reviewed.

Process for the Assessment of Test Materials in Nonanimal Methods

A clearly defined testing process is the central element in a nonanimal testing framework. These processes usually take the form of flow charts showing the key decision points, data-gathering procedures, and test methods that may be conducted during a safety assess-

ment. An example suitable for eye-irritation testing is shown in Figure 4. The process begins with the entry of a test substance into a safety assessment program. The first step in the process involves gathering as much previously existing information as possible about the material. The information obtained should include all available toxicity data on the test article, such as in vivo and in vitro data, human clinical data, supplier information, results from quantitative structure activity relationship (QSAR) analyses, physical-chemical data, marketplace experiences, and data on consumer habits and practices. In the case of completely new chemicals or formulations, information on similar materials should be gathered. Once these data are obtained, they must be assessed to determine if it is possible to complete the safety assessment without further testing. At this point, three decisions are possible: 1) market the product because the pre-existing data are considered adequate to support the product safety without further testing, 2) terminate or reformulate because the pre-existing data indicate the article is not safe for intended use, or 3) conduct additional testing because more data are necessary in order to complete the assessment. When the third decision is made, the next step in the process is to evaluate the article in an appropriate in vitro test(s). When the testing is complete, the results are passed through the algorithms of the prediction model so that a toxicity prediction can be obtained. The toxicity prediction is then considered along with the previously existing results. At this point it is again necessary to ask whether the test article is considered safe for intended use. If the answer is no, then reformulation or termination are the available options. If the answer is yes, it is necessary to decide whether human tolerance testing is necessary. Such studies may be needed, for example, to develop data for marketing claims support. If there is no need for human tolerance testing, then the safety assessment is completed.

Protocols and SOPs

Each safety assessment process contains several different tests. In order to facilitate the generation of reliable data from these tests, it is essential that all factors important to their conduct are clearly documented. It is therefore important that protocols and SOPs be provided for each test used in the safety assessment process. Adequate protocols and SOPs will contain at least four key elements. First, each SOP must have a detailed step-by-step description of how to conduct a test. Enough details need to be provided such that any appropriately trained and competent laboratory technician need use only this document as the guide to conduct the assay. Secondly, the SOP must indicate the steps used to define the final endpoint of the assay and the number of replicates necessary. Any data transformation or algorithms applied to the data should be clearly documented and consistently applied. Thirdly, the protocol should specify the positive and negative controls to be performed concurrently with each assay and the acceptable ranges for the resulting responses. Assays where the positive or negative controls values fall outside of those specified ranges would be considered invalid and should be repeated. Finally, the protocol must specifically describe the prediction model used to guide the interpretation of results.

Prediction Models

In order to use an in vitro test method in the safety assessment process, it must be possible to convert the in vitro results into a meaningful prediction of toxicity. The tool that is used to make this conversion is the prediction model [25,91]. A prediction model is considered adequate when it defines four elements. These elements include 1) a definition of the specific purpose(s) for which the alternative method is to be used, 2) a definition of all the possible results that may be obtained from an alternative method (inputs), 3) an algo-

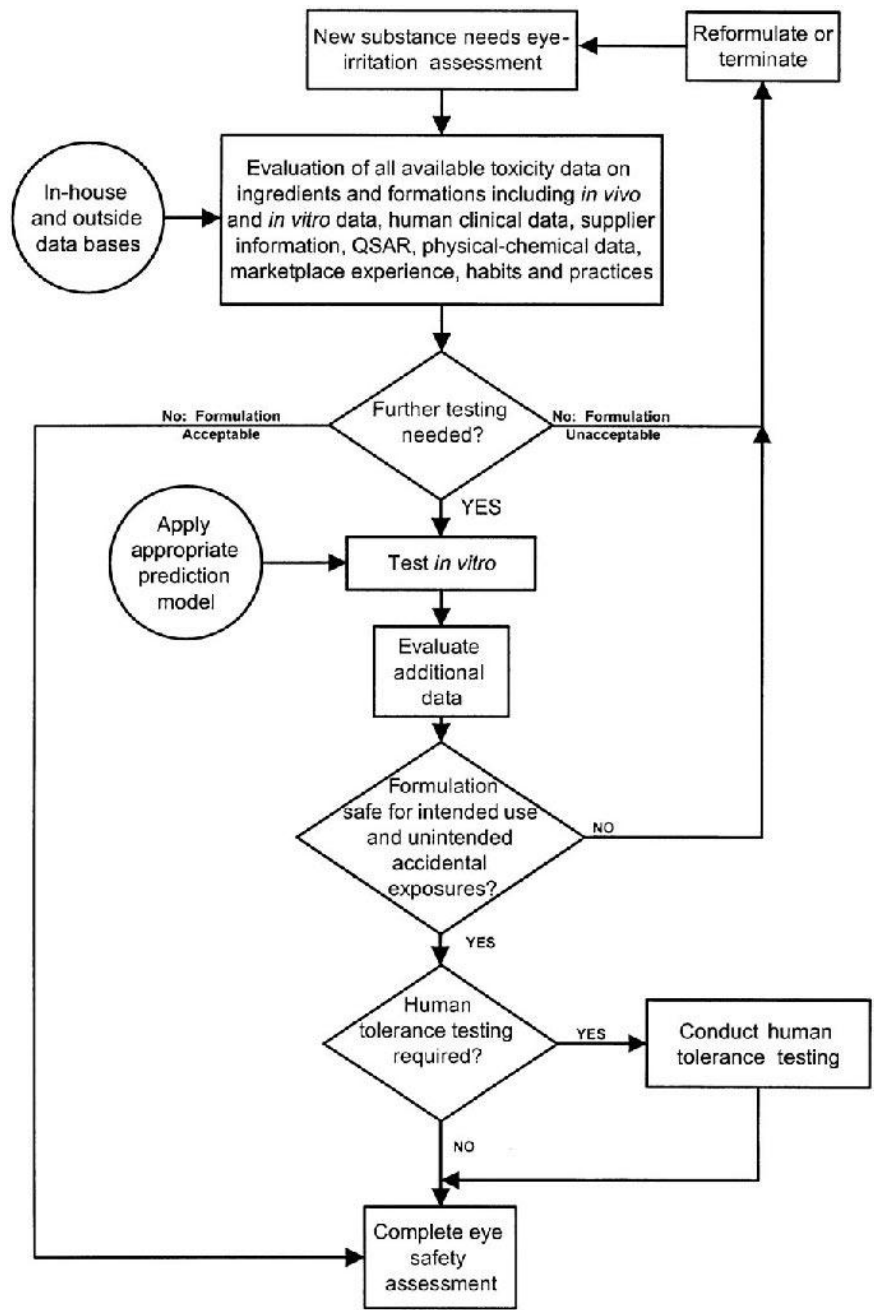

FIGURE 4 **Typical eye-irritation assessment process using nonanimal test methods.**

rithm that defines how to convert each alternative method result into a prediction of the in vivo toxicity endpoint (outputs), and 4) an indication of the accuracy and precision of outputs obtained from the model. An example of a prediction model for the Cytosensor microphysiometer is given in Figure 5. This figure shows the relationship between the in vitro test result (abscissa) and the predicted in vivo eye-irritation score (ordinate). The regression line fit to the data is shown running through the center of the data set and the upper 95% prediction interval is shown running through the upper periphery of the data. This model is useful when the test articles are surfactant-containing liquids.

Summary Document

The last element included in a nonanimal testing framework is a summary document. The purpose of this document is to advise toxicologists on the practical aspects of completing a safety assessment using the process previously described. These documents provide guidance on the test methods available for given classes of test substances, advice on

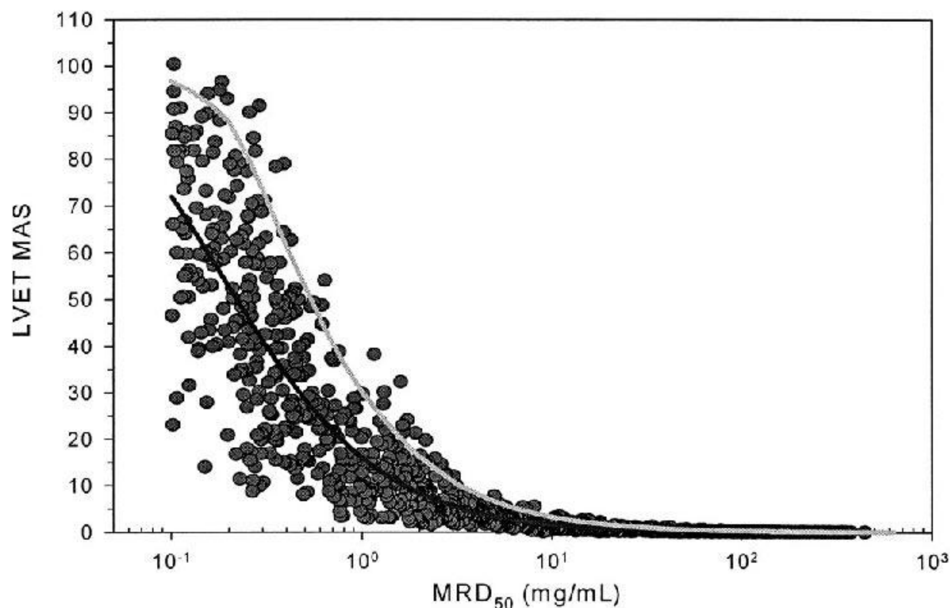

FIGURE 5 Cytosensor Microphysiometer prediction model. Prediction models are tools that allow the conversion of results from nonanimal tests into predictions of toxicity in vivo. The in vitro scores from the Cytosensor Microphysiometer are shown on the abscissa and the predicted in vivo scores, in terms of the low-volume eye-irritation test maximum average score (LVET MAS), are shown on the ordinate. The regression line running through the center of the data was derived by comparing the actual LVET MAS with corresponding data obtained from the same test substances evaluated in the Cytosensor Microphysiometer. Computer modeling was then used to simulate the data points shown in the plot and to generate the upper 95% confidence interval for predicting the LVET MAS from a cytosensor score (line running through the upper-right side of the data set). Models like the one depicted can be used to convert Cytosensor Microphysiometer scores into predictions of the LVET MAS with the indicated confidence as long as the test substance belongs to the same class as was used to develop the model. (From Ref. 25.)

which test should be used with different types of test substances, and an indication of the most appropriate prediction model to use with the test substance(s) being evaluated. Finally, these documents provide a wide range of relevant information on the technical aspects of the safety testing process (see the following) and names of individuals within the organization who can provide advice. All of this information is placed in a readily available location so that toxicologists can use the reference material easily.

Practical Considerations in the Conduct of Eye-Safety Assessments Without Animal Testing

In addition to establishing a framework for the practical conduct of eye-safety programs, it is also important to address several important technical issues that need to be considered when conducting in vitro tests. These matters have considerable influence on the choice of nonanimal tests to be used and the interpretation of the results. The matters that need to be considered include (1) the physical characteristics of the test article, (2) the expected toxicity of the test article, (3) the level of resolution required from the testing, and (4) resources available for a safety program.

Physical Characteristics of the Test Article. One of the most important considerations in the conduct of an in vitro test is the compatibility of the test article with the in vitro test being conducted. There are two general forms of in vitro tests: dilution-based tests where the target cells are completely immersed in growth medium, and topical application tests where the target cell surface is available for direct application of the test material (Table 2). For in vitro tests of the first type, it is necessary to serially dilute the test substance into a water-based cell culture medium and then apply the diluted test articles to the target cells. Dilution-based tests are particularly well suited for screening large numbers of water-soluble test substances quickly at a relatively low cost. The dilution-based tests also appear to have an increased capacity to distinguish between different degrees of mildness compared with the topical application tests [92].

Despite these advantages, the dilution of test articles in cell culture media results in technical problems that need to be considered before the procedure is used. First, because water-insoluble test substances cannot be diluted easily in aqueous cell culture media, it is generally unwise to evaluate water-insoluble substances in dilution-based tests. Second, when diluting test substances it is important to note that the dilution process can

TABLE 2 Dilution-Based and Topical Application–Based Assays: Examples of Dilution-Based and Topical Application–Based Assays Are Shown. Dilution-Based Tests Are More Suited for Test Substances That Are Water Soluble. Topical Application–Based Tests Have the Advantage That Dilution of Test Substance Is Not Required, Which Alleviates Technical Problems That Can Arise After Dilution. See Text for Details.

Dilution-based tests	Topical application–based tests
Cytosensor microphysiometer	Bovine corneal opacity and permeability assay
Fluorescein leakage test	Chicken enucleated eye test
Neutral red release test	Corneal and dermal 3-dimensional culture-based tests
Neutral red uptake test	Hen's egg test-chorioallantoic membrane (HET-CAM)
Red blood cell lysis test	Isolated rabbit eye test
Chorioallantoic membrane vascular assay (CAMVA)	

TABLE 3 Advantages and Disadvantages of Dilution-Based Assays. See Text for Details.

Advantages of dilution-based tests	Disadvantages of dilution-based tests
Rapid to execute	Cannot be used easily with water insoluble test substances
Most are machine scored	Dilution may mask toxicity of neat test substances
Generally very cost effective	The physical form of the test substance is changed
Work well with surfactants	Buffering may affect test substance toxicity
Often differentiate between mild test substances	Test substance may react with the diluent

significantly change the physical-chemical characteristics of a test substance. For example, the structure of complex emulsions can be changed dramatically by dilution in cell culture media. Crossing the critical micelle concentrations (CMC) for surfactants can change the toxicity observed. Dilution often changes the pH of a test article. If the irritant properties of a test substance in vivo are dependent on any factors such as physical form, micelle dissolution/formation, or pH, then the dilution of a test article may result in unreliable predictions from the in vitro test.

Topical application assays have a considerable advantage over dilution-based tests in that they are suitable for testing both water-soluble and insoluble test substances. Also, test articles can be assessed in exactly the same form as they were tested in vivo, thereby alleviating the technical concerns associated with dilution. Problems associated with topical application–based tests usually arise from the source and/or complexity of the target substrate. The use of abattoir-derived tissues may introduce variability into the results obtained from tests like IRE, CEET, and BCOP because of the random source of the animals. Also, because of the difficulties in producing large amounts of consistent substrate, the production of the 3-dimensional culture systems has most commonly been undertaken by commercial suppliers. These substrates therefore tend to be considerably more expensive than abattoir-derived tissues. It is necessary to carefully monitor the quality of commercial substrates to assure a consistent product. Withdrawal of product by several commercial suppliers in the past has also been a problem. The advantages and disadvantages of dilution- and topical application–based tests are summarized in Tables 3 and 4.

TABLE 4 Advantages and Disadvantages of Topical Application Assays. See Text for Details.

Advantages of topical application tests	Disadvantages of topical application tests
Material is tested in the same form as in vivo.	Test substrate is often expensive.
Exposure of the target tissue independent of solubility.	Exposure times may be inconveniently long.
In some models, exposure time can be selected to match expected in vivo exposure.	

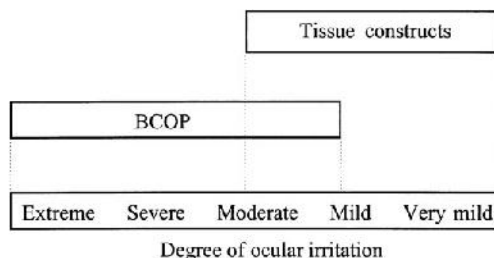

FIGURE 6 A diagram illustrating the relative sensitivity of the bovine cornea opacity and permeability test (BCOP) and 3-dimensional tissue constructs across the eye-irritation scale. Tissue constructs are most effective at the milder end and isolated eye and BCOP appear to be more suited for testing stronger irritants.

Toxicity Expected and Resolution Required. Another consideration in the choice of which in vitro test to use in a given situation is the expected level of toxicity possessed by the test material. Ocular toxicity ranges from very slight irritation to full corrosive destruction of eye tissues. Given this diversity of response it has been found that the results from single in vitro tests are incapable of reliably predicting irritation across the entire range of response. Experience has shown that the choice of in vitro assays must therefore balance between the resolution obtained from a test and its dynamic range. Topical application assays based on tissue constructs provide poorer resolution for more aggressive test articles that can kill cell cultures within a few seconds. In contrast, the bovine cornea does not resolve very mild products without excessively long exposures [49]. However, it has the robustness to discriminate at the medium to high end of the eye-irritation response [48]. Therefore, it is best to use tissue construct models if the expected irritancy of the test article is low to moderate. Models like the BCOP, IRE, and CEET are more appropriate for test substances thought to be of moderate or greater irritancy (Fig. 6).

Resources Available. The choice of which test to use also depends to some extent on the resources available for a given project. As previously noted, the cost of the different test methods varies considerably depending on the time required, the need for proprietary commercial substrates, and the equipment needed to conduct the test. It is often wise to use cheaper, less precise methods when large numbers of test substances need to be screened. Once the most promising candidates are identified, a limited number of definitive studies can be carried out using more definitive nonanimal tests that might involve more time and cost.

LOOKING TO THE FUTURE: WHERE DO WE GO FROM HERE?

Considerable progress has been made in the development of nonanimal methods for eye-irritation testing. These tests are increasingly used by industrial toxicologists in conjunction with previously existing in vivo data on benchmark formulations to help complete eye safety assessments of finished products. This progress has made it possible, for example, to support the elimination of in vivo eye-irritation testing of cosmetic finished products.

Despite the success with finished product testing, the progress observed with testing chemicals has been much more limited. The results of two large international validation studies illustrate the problems encountered. The first study, sponsored by the British Home Office and the European Commission through the European Centre for the Validation of Alternative Methods (ECVAM), evaluated nine in vitro methods using a set of 60 chemicals of known eye-irritation potential. The results from this study showed none of the tests could adequately predict eye-irritation responses of chemicals [70]. The second study, sponsored by the European Cosmetic Toiletry and Perfumery Association (COLIPA), evaluated 10 in vitro methods. The results were the same: current in vitro methods did not adequately predict the eye-irritation response of single chemicals [62].

Likewise, results from smaller studies on in vitro eye-irritation tests have not provided significant evidence that current nonanimal methods can fully replace the Draize eye-irritation test. In Germany, a study of the HET–CAM and the Neutral Red Uptake Test did not show that these assays could replace the in vivo eye-irritation test [88,89]. The results from the Japanese Ministry of Health and Welfare and the Japanese Cosmetic Industry Association suggested that several cytotoxicity tests were useful for testing a range of surfactant solutions, but more data would be needed to extend conclusions beyond this class of test substances [93].

Considerable analysis of the data from these validation studies has now been conducted in order to determine why the in vitro methods have been found insufficient for testing chemicals. The first major review of results from these efforts took place during an international workshop on nonanimal eye-irritation test methods in Brighton, United Kingdom [94]. The workshop panelists concluded that there are two likely explanations for the outcome: 1) the mechanistic understanding of current nonanimal methods has not been fully established, and 2) there are several parts of the eye-injury response that current in vitro tests do not assess. In addition to the Brighton Workshop, an ECVAM Task Force on eye-irritation testing reviewed the results of recently completed validation studies and made recommendations on the way forward [3]. The authors of the report concluded that further refinement of current methods might improve them for use as screening tests. However, because current in vitro tests cannot yet replace animal tests for assessing chemical irritancy, there is a need for additional research leading to improved understanding of eye-irritation mechanisms.

Mechanistic Basis for the Development of Nonanimal Replacements for the Draize Eye-Irritation Test

Attempts to validate a nonanimal replacement for the in vivo eye-irritation test have principally been by correlative analyses using information derived from the Draize scoring scheme. As can be seen in Table 1, the assessment of the eye-irritation response scoring is based on subjective visual observations made by a technician aided with a pen light. This approach to the measurement of in vivo eye-irritation responses does not provide insight into the primary and secondary pathophysiological responses occurring in the cornea, iris, or conjunctiva after chemical injury [15,95–98]. The subjective observations used in the Draize scoring scheme also provide little information on the differences in the underlying pathological changes associated with scores obtained across the time-course of an eye-irritation test [15,95–100]. For example, a high score occurring very early in an eye-irritation test is more likely reflective of the primary damage caused by a chemical, whereas a high score occurring later in a study more likely reflects secondary inflammatory

responses developing in response to the primary injury. Overall, these observations suggest that the scoring system used in the current in vivo eye-irritation test may not provide enough information about the critical cellular and molecular changes involved in ocular injury and repair to be used as the basis for developing adequately predictive nonanimal tests.

In order to address this shortcoming it has been proposed that more data must be obtained on the pathophysiological processes underlying chemical-induced eye-irritation responses [99]. The new information needs to be derived from additional in vivo testing of a panel of test substances covering relevant chemical classes and the appropriate range of eye-irritation response. Where possible, these studies should include test substances for which there is human eye-irritation data so that alternative methods can be developed to predict human responses [101]. The information derived from the new in vivo studies would characterize the key cellular and molecular events and extent of variability occurring with the ocular irritation response and serve as the basis for the development mechanism–based replacement tests [15,95–100]. Preliminary work suggests that two areas of research are likely to be most beneficial. These include studies to (1) characterize the pathological changes associated with the initial eye injury caused by chemical, and (2) characterize changes in the expression of cytokines and other extracellular factors associated with inflammation and corneal repair.

Characterizing the Pathological Changes Associated with Initial Eye Injury

Differences in extent of the initial tissue injury after chemical exposure has been hypothesized to be one of the primary factors that determine the responses and ultimately the final outcome of an ocular irritation response [15,96–100]. Results from studies of a broad sampling of surfactants support this premise [15,95–100,102–104]. Light microscopy [15,97,99] and in vivo confocal microscopy [95,96,102] studies in rats and rabbits show there are differences in the extent of ocular injury induced by surfactants of known irritancy occurring as early as 3 hours after treatment. Collectively, these studies indicate that slight irritants affect only the superficial corneal epithelium, mild and moderate irritants affect the epithelium and superficial stroma, and severe irritants affect the epithelium, deep stroma, and at times the endothelium. Additional work suggests that the extent of surfactant-induced injury correlates with cell death [98] and that the extent of the primary injury correlates with subsequent responses and the eventual outcome in rats [97] and rabbits [100].

Overall, these results suggest that prediction models for mechanism-based in vitro tests could be developed based on measurements of the extent of injury and, perhaps more specifically, on measures of cell death in the cornea after chemical treatment in vivo [98,100]. Such an approach would require that replacement tests assess the area and depth of injury in multilayered in vitro substrates that contain at least a stratified epithelium and keratocyte-laden stroma [98]. Examples of appropriate substrates for such studies include isolated whole eye and isolated cornea models, or perhaps 3-dimensional corneal models like those previously described [98].

Characterizing Changes in Expression of Cytokines and Other Extracellular Factors

Changes in the expression and/or levels of biomarkers, cytokines, and other extracellular factors associated with the different stages of chemically induced eye injury have also

been proposed as possible endpoints for mechanism-based replacement [15,96,97,105]. For example, Sotozono et al. [106] have observed that the production of IL-1α and IL-6 reflect the severity of alkali burns on the cornea. Shams et al. [107] have shown that levels of corneal IL-1 correlate with severity of inflammation. Planck et al. [108] have proposed that cytokine signatures characterized by varying patterns of expression of biological factors occur with different types of corneal injury. In this regard, their studies in rats have indicated IL-6 induction occurs with alkali burns and incisional trauma of the cornea, whereas IL-1β induction occurs with alkali burns but not incisional trauma. Further, differences in mRNA expression for different chemokines were observed in mouse corneas infected with HSV-1 versus traumatic injury [109]. Finally, a more recent study has indicated that differences in expression of corneal IL-1α, IL-1β, and IL-6 levels are observed after surfactant-induced injury in rats with the magnitude of the differences reflecting the extent of injury observed [105].

Other Endpoints Worthy of Consideration

In addition to studies of pathology and inflammatory mediator release associated with chemical injury, there are other areas of research that may be of interest. First, it may be useful to examine other early events occurring after exposure of the eye to chemicals. Studies could explore the interaction of chemicals with cell membranes that lead to acute damage of the eye tissue and activation of ocular nerves. Approaches that may be useful for such work include quantitative structure-activity relationship approaches and neurophysiological models of the eye [110]. After the initial chemical trauma, various physiological responses in addition to inflammatory mediator release take place in the intermediate stages of the response depending on the extent of the initial damage and the modulating influence of nerve activation. Therefore studies on the physiological effects of chemicals on isolated eyes may prove useful. In the later stages of the reaction, the inflammation subsides and the eye returns to a quiet state. Of critical importance is whether or not the eye returns to the normal pre-exposure state or whether there is scarring of the cornea that can lead to vision deficit or, in the worst case, loss of sight. Therefore, the biological responses related to recovery need to be studied. As these areas are evaluated in ongoing research programs sponsored by industry and relevant governmental agencies, the new knowledge gained may be directly applied to the development of mechanism-based assays that may be validated by interested parties.

CONCLUSION

Nonanimal test methods are now routinely used by industrial toxicologists to assess the safety of certain test articles [111]. These tests are most useful when conducted as part of a larger process that uses significant amounts of other supporting information. No single test or battery of tests can yet completely replace the need for animals in ocular safety testing. If complete elimination of animal use in eye safety assessment is to be achieved, a better understanding of the mechanisms by which chemicals cause eye irritation will be needed. The areas of research needed have been outlined in considerable detail and proposals have been made for the conduct of the research. The application of recent progress in tissue-culture techniques, cellular and molecular biology, and analytical cytometric techniques will greatly facilitate the conduct of this research and lead us closer to our ultimate goal of eliminating the need for animals in ocular safety testing.

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