41 Classification of Irritant Contact Dermatitis

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INTRODUCTION

Contact dermatitis is defined as inflammation of the skin invoked as a result of exposure to an exogenous agent and constitutes a key portion of occupational disorders in industrialized societies.

In 1898, contact dermatitis was first appreciated to have more than one mechanism and is now generally divided into *irritant contact dermatitis* (ICD) and *allergic contact dermatitis* (ACD) on the basis of these mechanistic differences. ACD is a delayed (type IV) hypersensitivity reaction, mediated by T cells and requiring prior sensitization, while ICD has a non-immunological mechanism, thus not requiring sensitization. Clinical distinction of the two processes is often challenging, as morphology and histopathology of irritant and allergic dermatitis reactions can be virtually indistinguishable. The two processes may, and often do, coexist, thereby further complicating matters.

The morphological spectrum of ICD is broad and frequently impossible to distinguish from ACD and even endogenous (atopic) dermatitis. Chronological descriptions of these processes are often clinically used. "Acute, subacute, and chronic dermatitides" are terms applicable to ACD and ICD as well as atopic dermatitis. The erythema, edema, and vesiculation seen in acute dermatitis or the hyperkeratosis, lichenification, and fissuring seen in the chronic phase are largely nonspecific signs. Although chronological classification has its uses, the main classification of irritation is now based on both morphology and clinical course of the dermatitis.

CLINICAL CLASSIFICATION OF ICD

ICD (*synonyms: cutaneous irritation, irritant dermatitis*) is the biological response of the skin to a variety of external stimuli that induce skin inflammation without the production of specific antibodies. Formerly considered a monomorphous process, it is now understood to be a complex biological syndrome, with a diverse clinical appearance, pathophysiology, and natural history. The clinical appearance and course of ICD vary depending on multiple external and internal factors. This diversity in clinical presentation has generated a classification scheme, on the basis of both morphology and mode of onset. The various subtypes of ICD and their respective prognoses are tabulated in Table 1.

Acute ICD

When exposure is sufficient and the offending agent is potent, classic signs of acute skin irritation are seen. Erythema, edema, inflammation, and vesiculation are typical features, although acute irritation may range from mild erythema through exudative cutaneous inflammation to ulcerative lesions and frank epidermal necrosis, depending on factors such as the chemical and exposure time (1). At the extreme end of this spectrum is the "chemical burn"—this entity is recognized by severe tissue damage as a result of exposure to highly alkaline or acidic compounds—most often as a result of an industrial accident. Symptoms of acute ICD are pruritus, burning, stinging, and pain.

In keeping with an exogenous dermatosis, acute ICD usually exhibits an asymmetrical distribution and sharply demarcated borders. These borders delineate the area of exposure to the

Table 1 Ten Subtypes of ICD

	Irritation	Onset	Prognosis
1.	Acute ICD	Acute—often single exposure	Good
2.	Delayed acute ICD	Delayed—12–24 hr or longer	Good
3.	Irritant reaction	Acute—often multiple exposures	Good
4.	Chronic ICD	Slowly developing (wk–yr)	Variable
5.	Traumatic ICD	Slowly developing after preceding trauma	Variable
6.	Acneiform ICD	Moderately slowly developing (wk–mo)	Variable
7.	Nonerythematous (suberythematous) irritation	Slowly developing	Variable
8.	Subjective (sensory) irritation	Acute	Excellent
9.	Friction dermatitis	Slowly developing	Variable
10.	Asteatotic irritant eczema	Slowly developing	Variable

offending chemical. Contact with a potent irritant is often accidental, and an acute ICD is elicited in almost anyone, independent of constitutional susceptibility—in contrast to chronic ICD.

This classic, acutely developing dermatitis usually heals soon after exposure, assuming there is no reexposure—this is known as the "decrescendo phenomenon." In contrast, ACD usually exhibits a "crescendo phenomenon," i.e., transient worsening of symptoms and signs, despite removal of the allergen. In unusual cases, ICD may persist for months after exposure, followed by complete resolution.

The availability of the material safety data sheet and data from the single application Draize rabbit test combined with activities of industrial hygienists and other informed personnel have greatly decreased the frequency of such dermatitis in industry.

Delayed Acute ICD

Some chemicals produce acute irritation in a delayed manner so that inflammation is retarded until 8 to 24 hours or more after exposure (2). Except for the delayed onset, the clinical appearance and course resemble those of acute ICD. The delayed acute irritant dermatitis, because of its delayed onset and atypical crescendo periodicity, is often confused with ACD; appropriately performed diagnostic patch tests easily separate the two, i.e., the substances implicated in delayed, acute ICD would result in negative patch test results. In delayed acute ICD, a burning sensation predominates, rather than pruritus. Examples of substances causing delayed irritation are hexanediol and butanediol diacrylates (2), dithranol (anthralin), calcipotriol, and benzalkonium chloride.

Irritant Reaction

Individuals extensively exposed to irritants often develop erythematous, chapped skin in the first months of exposure. This irritant reaction may be considered a pre-eczematous expression of acute skin irritation. The term "irritant reaction" is now increasingly used if the clinical picture is monomorphic, rather than the usual polymorphic appearance of ICD, i.e., only one of the parameters usually seen in ICD are present, e.g., scaling, erythema, vesiculation, pustules, or erosions. This pattern is frequently seen in hairdressers and other wet workers. Frequently, this condition heals spontaneously, with hardening of the skin. However, repeated irritant reactions can sometimes lead to contact dermatitis, usually with good prognosis. Compounds that cause irritant reactions are typically mild irritants, such as detergents, soaps, and water.

Chronic ICD

When exposure inducing an acute irritant dermatitis is repeated, the dermatitis tends to persist and becomes chronic (more than 6 weeks has been suggested as an arbitrary threshold period). In chronic ICD (*synonyms: cumulative ICD, traumiterative dermatitis, wear and tear dermatitis*), the frequency of exposure is too high in relation to the skin recovery time.

Multiple subthreshold skin insults lead to a manifest dermatitis when the irritant load exceeds the individual's elicitation threshold for visible effects. Chronic ICD was called "traumiterative dermatitis" in the older German literature ("traumiterative" = traumas repeating) (3,4). Classic signs are erythema and increasing xerosis (dryness), followed by hyperkeratosis with frequent fissuring and occasional erythema. The lesions are usually localized but ill

defined. Pruritus and pain due to fissures are symptoms of chronic ICD. Chronic ICD often presents as hand eczema ("housewives" eczema').

Chronic ICD is the most common type of ICD. This clinical picture may develop after days, weeks, or years of subtle exposure to chemical substances. Variation in individual susceptibility and the physical properties of the irritating substance increase the multiplicity of clinical findings. Delayed onset and variable attack lead to confusion with ACD. To rule out an allergic etiology, appropriate diagnostic patch testing is indicated. Models of chronic ICD have been developed, contributing to product evaluation and mechanistic insights (5,6).

Traumatic ICD

Traumatic ICD develops after acute skin trauma, such as burns, lacerations, or acute ICD. The skin does not completely heal, but erythema, vesicles, papules, and scaling appear at the site of injury. The clinical course later resembles discoid (nummular) dermatitis. It may be compounded by a concurrent allergen exposure. The healing period is generally prolonged.

Often these patients are considered to have factitial dermatitis because of a healing phase followed by exacerbation. Although factitial aspects may occur in some patients, this peculiar form of irritation appears to be a disease sui generis. Its chronicity and recalcitrance to therapy provides a challenge to both patient and physician.

Acneiform ICD

Certain exogenous substances have the capacity to elicit an acneiform eruption (7,8), and even allergic reactions may sometimes be pustular or follicular (9). Acneiform ICD (*synonyms: pustular ICD, follicular ICD*) should always be considered in the differential diagnosis of an adult with acneiform lesions. The pustules are usually sterile and transient.

In occupational exposure, only a minority of subjects develop pustular or acneiform dermatitis. Thus, the development of this type of ICD appears to be dependent on both constitutional and chemical factors. *Chloracne* is an industrial disease caused by exposure to chlorinated aromatic hydrocarbons, in particular chlorinated dioxins, which are the most potent acnegenic agents. Many of the chloracnegens are also hepatotoxic; therefore, this is a disease of medical importance. Acneiform ICD may also develop from exposure to metals, mineral oils, greases, tar, asphalt, cutting oils, and metalworking fluids.

Acne cosmetica represents acneiform ICD caused by cosmetics. *Pomade acne* is a well-known form of acne cosmetica, seen in Afro-Caribbean women who apply vegetable oils to their skin (10). A similar problem has been reported with applications of white petrolatum (11). Nowadays, most cosmetics available in Western countries are noncomedogenic and nonacnegenic.

Non-erythematous or Suberythematous Irritation

In the early stages of skin irritation, subtle skin damage may occur without visible inflammation. As a correlate of non-visible irritation, objectively registered alterations in the damaged epidermis have been reported via cutaneous bioengineering techniques (12–14). It is customary in Japan to screen new chemicals, cosmetics, and textiles for subtle signs of stratum corneum (SC) damage, employing replicas of SC (the Kawai method; Kawai 1971). A similar technique, squamometry or corneosurfametry, has now been refined to detect subtle subclinical alterations in the SC caused by application of mild irritants (15).

Subjective or Sensory Irritation

Some individuals ("stingers") experience itching, stinging, burning, or tingling sensations on contact with certain chemicals (14,16), despite a distinct lack of objective signs on clinical examination. Despite the lack of clinical manifestations, the subjective sensations are reproducible, typically occurring within seconds to minutes following exposure; this type of irritation is known as subjective or sensory irritation. Lactic acid is a model for this non-visible cutaneous irritation. The threshold for this reaction varies between subjects, independent of susceptibility to other irritation types. The quality as well as the concentration of the exposing agent is also important, and neural pathways may be contributory, but the pathomechanism is unknown. Some sensory irritation may be subclinical contact urticaria. Screening raw ingredients and final formulations in the guinea pig ear swelling test (17) or the human forehead assay allows us to minimize the amount of subclinical contact urticaria.

Although subjective irritation may have a neural component, recent studies suggest that cutaneous vasculature may be more responsive in stingers than non-stingers (14,18). At least 10% of women complain of stinging with certain facial products; thus, further work is needed to develop a strategy to overcome this type of discomfort.

Friction Dermatitis

Repeated friction of low intensity is known to induce callus formation (hyperkeratosis and acanthosis), hardening of the skin, hyperpigmentation, and friction blisters in normal skin. In atopic people, lichenification and lichen simplex chronicus may ensue a result of friction. All of the above may be considered as adaptive phenomena to friction and should not be confused with friction dermatitis.

True friction dermatitis is the development of ICD in response to low-grade friction—this is seen clinically as erythema, scaling, fissuring, and itching surrounding the area of frictional contact. The syndrome has been characterized by Susten (19). Cases of occupational friction dermatitis in the literature are seldom documented, but most often reported in association with paper work (20). More recently, a short collection of further cases of friction dermatitis has been published (21).

Asteatotic Irritant Eczema

Asteatotic eczema (*synonyms: asteatotic dermatitis, exsiccation eczematid, eczema cracquele*) is a variant of ICD seen in elderly individuals, as a result of worsening xerosis, particularly during dry winter months. Clinically, the skin is dry (xerosis), with loss of smoothness, ichthyosiform scale, and cracking of the superficial epidermal layers, often associated with eczematous changes. The term "eczema cracquele" refers to the cracked, patchy eczematous appearance (likened to cracked porcelain, or "crazy paving") usually seen on the lower legs of these individuals. An uncomfortable sensation of "tightness" and pruritus is often felt.

Xerosis is a result of low water content in the SC, causing the SC to lose its suppleness and the corneocytes to be shed in large polygonal scales. Xerosis is usually more pronounced in the elderly and in atopic individuals. Environmental insults, such as low humidity, low temperatures, and very high doses of ultraviolet radiation (UVR) [>3 or 4 minimal erythema doses (MEDs)] can help accelerate this process. In an occupational setting, this is sometimes combined with repeated exposure to wet work, chemical insults, and friction, cumulating in perturbation of the skin barrier. Skin barrier dysfunction then leaves the skin even more vulnerable to exogenous insults and asteatotic irritant eczema ensues.

Miscellaneous

Airborne ICD is not included as one of the 10 genotypes as the mechanisms are similar to acute or chronic ICD; the only difference is that the irritant substance is dispersed and transported in the air before contact with skin. This causes dermatitis on exposed areas of skin, most commonly on the face, and may mimic photoallergic reactions.

Phototoxicity or photoirritation is another form of skin irritation following cutaneous or systemic exposure to a phototoxic agent in combination with appropriate radiation (most often in the UVA spectrum). *Phytophotodermatitis* specifically represents phototoxic dermatitis in response to plants or plant derivatives, such as species in the *Umbelliferae* (e.g., celery, carrot) and Rutaceae (e.g., lime, lemon, bergamot) families. *Berloque dermatitis* refers to fragrance dermatitis because of bergapten, the photoactive compound found in oil of bergamot, an ingredient found in fragrances; this compound has now been removed from most perfumes and substituted with artificial or highly refined bergamot oil.

Other reactions, which can be caused by contact with irritant substances, but do not fall within the scope of this chapter, include pigmentary alterations, nonimmunological contact urticaria, granulomatous reactions, and alopecia.

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42 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) and individual susceptibility (2). The clinical appearance of ICD is extremely variable. It is determined by the type of irritant and a dose-effect relationship (3). The clinical morphology of acute ICD 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 (4–6).

Despite their different pathogenesis, ACD and ICD, particularly chronic conditions, show a remarkable similarity with respect to clinical appearance, histopathology (7,8), and immunohistology (9,10). Therefore, ICD can be regarded as an exclusion diagnosis after negative patch testing. The histological pattern of chronic ICD is characterized by hyper- and parakeratosis, spongiosis, exocytosis, moderate to marked acanthosis, and mononuclear perivascular infiltrates with increased mitotic activity (11,12).

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 (10). Nevertheless, the underlying pathophysiological mechanisms are thought to be substantially different (13). Attempts to differentiate the types of contact dermatitis with new methods are constantly under way. Recently, in vivo reflectance confocal microscopy has been suggested as an adjunctive tool in contact dermatitis diagnosis (14). With this technique, features of ACD and ICD that include spongiosis, exocytosis, vesicle formation, and blood vessel dilatation can be visualized. Hallmarks of ICD that are stratum corneum disruption, epidermal necrosis, and hyperproliferation, whereas ACD is supposed to present more typically with vesicle formation (14). However, these findings probably relate to acute dermatitis, whereas chronic allergic and irritant dermatitis can be expected to be undistinguishable by in vivo reflectance confocal microscopy just as they are by light microscopy.

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 (15). In a recently published study of Meller et al., it was demonstrated that chemokine responses are helpful characteristics to distinguish the chemical-induced allergic from the irritant skin inflammation. They found that allergic and irritant skin responses have distinct molecular expression profiles. Chemokine genes predominantly regulated by T-cell effector cytokines demonstrated differential upregulation in hapten-specific skin inflammation. CXCR3 ligands, such as CXCL9 and CXCL10, were selectively induced during hapten-specific, but not irritant-induced skin inflammation. It was demonstrated that effector cytokines released by a small number of activated hapten-specific memory T cells

stimulate gene expression of a large number of surrounding resident cells, leading to the production of a discriminative chemokine signature. In contrast, the absence of antigen-specific T-cell activation in irritant skin responses results in only negligible amounts of T-cell-derived effector cytokines (16).

Epidermal keratinocytes play a crucial role in the inflammation of ICD; they can be induced to produce several cytokines and provoke a dose-dependent leukocyte attraction (17). In response to the impairment of stratum corneum barrier with direct toxic effect on keratinocytes (14,18,19), preformed IL-1α is released. It stimulates other keratinocytes and fibroblasts to produce more Il-1α, IL-1β, IL-6 IL-8, and TNF-α (13,20–23). The cytokine-induced cascade leads to vasodilatation in the dermis and cellular infiltrate in the epidermis (13,24,25). But keratinocytes also produce anti-inflammatory cytokines to counteract these inflammatory processes. The IL-1 receptor antagonist (IL-1RA) blocks IL-1 activity by competitive binding to the IL-1 receptor without triggering a signal cascade. IL-10 is another anti-inflammatory cytokine (20,23).

The upregulation of certain adhesion molecules like α6 integrin or contact dermatitis 36 is independent from the stimulus and not cytokine induced (18,26). 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, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TGF-α can promote growth. Other cytokines, such as IL-10, IL-12, and IL-18, are known to regulate humoral versus cellular immunity (27). It is still controversial whether the cytokine profile induced by irritants differs from that induced by allergens (28–31). In irritant reactions, TNF-α, IL-6, IL-lβ, and IL-2 have been reported to be increased (32,33). De Jongh et al. recently investigated stratum corneum cytokines and skin irritation responses after single and repetitive exposures to sodium lauryl sulfate (SLS) (34). They found an IL-1α decrease of 30% after repeated exposure, while IL-1RA increased 10-fold and IL-8 increased 4-fold. Baseline IL-1RA and IL-8 values after single exposure were predictors of transepidermal water loss (TEWL) and erythema. Their results suggest that subjects with higher baseline stratum corneum levels of IL-1RA and IL-8 have a stronger response to skin irritation and that baseline levels of these cytokines can serve as indicators of skin irritability (34).

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 TEWL. This is one triggering stimulus for lipid synthesis, and it promotes barrier restoration (35). Nevertheless, recent studies show that the concept of TEWL increase after SLS being directly related to a delipidizing effect of surfactants on the stratum corneum cannot be kept up without limitation. Moreover, SLS exposure for 24 hours causes damage in the deeper nucleated cells of the epidermis, leaving the lamellar arrangements of lipids intact (36).

However, lipids of the stratum corneum play an important role for barrier function. Proksch et al. observed an increase in skin lipid synthesis after acute irritation with acetone treatment (37,38). Heinemann et al. observed an upregulation of the production of ceramide 1 in response to repeated irritation with SLS, thus suggesting that this upregulation might play a major role in the development of a hardening phenomenon (39). The hardening effect is understood as the adaptation of skin to repeated exogenous irritative noxes clinically resulting in stabilized skin state in spite of ongoing irritant exposure.

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 (40,41). Disruption of the stratum corneum can stimulate cytokine production itself, and in this way promote the inflammatory skin reaction, as shown by Wood et al. (42). They found an increase of TNF-α, various interleukins, and GM-CSF.

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 mRNA expression for IL-6, whereas mRNA expression for GM-CSF was increased after SLS (43). 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 (44).

Other interesting details for understanding the molecular mechanisms of skin irritation have been contributed by Ma et al. (45). They investigated the role of metallothioneins (MTs) in SLS-induced skin irritation in MT genes I and II knockout mice [MT(–/–)] and demonstrated that MT (–/–) mice showed a much higher degree of skin inflammation than MT (+/+) mice did. With this result, they suggested that MT I and II genes presumably play an important role in skin irritation.

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 (46,47), females turned out to be at risk in some epidemiological studies (48,49). Presumably increased exposure to irritants at home, caring for children younger than four years, lack of dishwashing machine (50), and preference for high-risk occupations contribute to the higher incidence of ICD in females (47).

The most established individual risk factor of several epidemiological studies concerning irritant hand dermatitis, is atopic skin diathesis (48,51–54). On the other hand, experimental studies concerning the reactivity of atopics and nonatopics to standard irritants have given contradictory results (55,56) 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 (57). 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 (58,59). 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 (60–63).

Clinical manifestation of ICD is influenced by type and concentration of irritant, solubility, vehicle, and length of exposure (64) as well as temperature and mechanical stress. Pathogenesis of ICD is complex and may be related to a combination of different types of irritants as well as to different types of irritation. Sequential ("tandem") exposure to different irritants often occurs in the workplace and modifies the cutaneous response, in contrast to repeated exposure to each irritant alone, indicating a potential aggravating effect of the combination of chemically different irritants (65,66). In several studies, the synergistic or additive effect on skin response of irritants in combination was investigated (67). It has been demonstrated that the repeated sequential application of occlusion (with gloves, water, or SLS) and mechanical irritation enhances the effect on barrier disruption caused by single application (68). It was also described that concurrent application of an anionic detergent and a mild acidic irritant can lead to disruption of the barrier function, which, although not additive, is still considerable. The combined application of SLS and mild acids (ascorbic and acidic acid) did not prevent SLS-induced irritation. NaOH in low concentrations may also act as a potent irritant, but its effect is not enhanced by SLS (69). The contact with substances that are potentially barrier disruptive, especially in combination with other irritants, boosts the susceptibility for ICD. In contrast, exposure to low concentrations of organic fruit acids either alone or in combination with SLS did not significantly contribute to the development of ICD or increase susceptibility to SLS-induced irritation (70).

Changes in climatic conditions are known to influence barrier function and to induce ICD (71–76) or to aggravate preexisting skin irritation. Sequential treatment with airflow and SLS led to an impairment of barrier function and irritation stronger than caused by SLS alone (76). Similar effects might occur under low humidity conditions, which are known to desiccate skin, such as during the winter months (73,74,77,78).

EPIDEMIOLOGY

Population-based data on the incidence and prevalence of ICD are rare. The figures on the incidence of ICD vary considerably, depending on the study population. Most data stem from studies about occupational hand dermatoses.

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 one- to three-year period prevalence rates of 6.2% to 10.6% (79).

An extensive study of Meding on hand eczema in Gothenburg, Sweden, included 20,000 individuals randomly selected from the population register (48). She estimated a one-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 (48,49). 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, and 10% micellaneous diagnoses (80). Even still higher rates of ICD were found by Soder et al. in cleaning and kitchen employees. One hundred and sixty-eight (79.2%) of 212 participants suffered from hand dermatitis, and ICD with 46.2% ($n = 98$) was the predominant diagnosis (81). A Danish study on occupational hand eczema revealed rates of 61.9% for ICD and 21.2% for ACD (82). The proportion of occupational ICD was similar for males and females (59.7% and 63.1%, respectively), even though females were overrepresented in wet occupations (83). In accordance with these findings, a retrospective epidemiological study of occupational skin disease in Singapore over a two-year period also demonstrated that ICD is more common than ACD: ICD made up for 62.4% of all cases of occupational contact dermatitis, ACD constituted 37.6% (84).

Interesting findings result from investigations of the severity of irritant hand dermatitis five years after initial diagnosis (85). Fifty percent of 124 ICD cases had still medium and 32% severe hand dermatitis demonstrating that irritant hand dermatitis is chronic in duration. Skoet et al. found a mean disease duration of 4.4 years for males and 4.9 years for females (83).

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 (86). Adverse reactions to cosmetics and hygiene products occur predominantly in females (87). Clinical examinations have revealed that the majority of self-reported reactions are of irritant type (88,89). Most untoward reactions caused by cosmetics occur on the face, including the periorbital area (90).

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 (91). In Sweden, the top-ranking products causing adverse effects, as reported by the Swedish Medical Products Agency, were moisturizers, hair care products, and nail products (87). In a Danish population survey with persons aged 19 to 80 years asked for self-diagnosed dermatitis, the reported one-year prevalence of skin symptoms on the face (acne excluded) was 14%. Of those who reported skin symptoms on the face, 33% also reported hypersensitivity to cosmetics (92).

In other studies, the incidence of cosmetic intolerance varied between 2.0% and 8.3%, depending on the test population (90,93,94). 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 (95). Since most consumers just stop using cosmetics and hygiene products when experiencing mild irritant or adverse reactions and seldom consult a physician, it can be assumed that mild irritant reactions to cosmetic products are still underestimated (92,96).

The symptoms of discomfort such as stinging, burning, itching noticed by many persons following product applications are summarized in the term "sensitive skin." Only little epidemiological evidence exists with respect to its prevalence. In 2001, Willis et al. (97) published an epidemiological study in the United Kingdom to assess the prevalence of sensitive skin in the population and to examine possible factors that may be associated with sensitive skin. They found that sensitive skin is a common phenomenon with about 50% of women and 40% of men regarding themselves as having a sensitive skin. 10% of women and

5.8% of men described themselves as having very sensitive skin. Jourdain et al. (98) reported that 52% of women aged between 18 and 45 years agreed with the statement: "I have a sensitive facial skin." Approximately 30% of the total population strongly agreed with this statement.

CLINICAL TYPES OF ICD

According to the highly variable clinical picture, several different forms of ICD have been defined. The following types of irritation have been described (15,99):

- 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 ICD and ACD 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 (100). 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 result 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 (101). Tetraethylene glycol diacrylate caused delayed skin irritation after 12 to 36 hours in several workers in a plant manufacturing acrylated chemicals (102).

Irritant Reaction

Irritants may produce cutaneous reactions that do not meet the clinical definition of "dermatitis." An 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 (103). 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 (99). 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 (104). 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 (105). Vesicles are less frequent in comparison to allergic and atopic types (48); 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 (47,83,85,106–108). Some investigators suggest that the repair capacity of the skin may enter a self-perpetuating cycle (104).

Traumiterative ICD

The term "traumiterative ICD" has often been used similarly to cumulative ICD in the past (99,103). 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 (3).

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 bums, 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 of this rare type of ICD resembles that of nummular dermatitis (99).

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 and locations. Patients with seborrhoea, macroporous skin, and prior acne vulgaris are predisposed along with atopics.

Nonerythematous ICD

Nonerythematous ICD is an early subclinical 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 (99,109).

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 minutes/hours after contact. Those individuals with

hyperreactive 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 (110,111). 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 (111). 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 (111,112). Several investigators tried to determine parameters that characterize those individuals with "sensitive skin,"s a term that still lacks a unique definition (113,114). 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 TEWL, and higher pH values accompanied by lower sebum levels (114–116). 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, enhanced immune responsiveness, and increased sweat glands (113,117,118). 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 (119). Detailed recommendations for formulation of skin care products for sensitive skin have been given by Draelos (113). Recent reviews on experimental studies on the nature of sensitive skin and on host factors were published by Kligman et al. (120) and Farage et al. (116) in 2006.

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43 Mechanism of Skin Irritation by Surfactants and Anti-Irritants[a] for Surfactant-Based Products

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INTRODUCTION

Each day our skin is in contact with a multitude of aggressions that we need to minimize. This can be done by decreasing the intrinsic irritation potential of the insult, by placing an additional barrier between the irritant and our skin, or by changing our behavior. Chemical irritants are usually the best-known irritants that inflame our skin, but physical, biological, and environmental factors are also important causes of irritation (Table 1)

In some cases, several irritant categories may act simultaneously on the skin to potentiate their effect. For instance, scrubbing products involve a mechanical stress of the skin by rubbing the skin with solid particles and a chemical stress by the surfactants used to formulate the vehicle. With so many types of potential irritants, it is obvious that skin irritation can be induced through different pathways.

SURFACTANTS

Surfactants: A Good Model to Investigate Skin Irritation

Surfactants are frequently used as a model to investigate skin irritation and the effect of anti-irritants for three main reasons.

Surfactants are a Major Cause of Skin Irritation

As a result of their detergent and foaming properties, surfactants find broad use in many domestic products that contact the skin (Table 2). Furthermore, many subjects take several showers/baths a day for cleansing as well as for relaxation and pleasure.

It is Quite Easy to Obtain Very Well-Standardized Surfactants to Work With

In the scientific literature, sodium lauryl sulfate (SLS) is regularly used as the "gold" standard to induce skin irritation (18) for several reasons:

- SLS is classified as a skin irritant, Xi-R38 (19).
- SLS can be obtained in a very pure form, which allows different laboratories to work on the same material.
- SLS can be easily formulated in various vehicles.
- Allergic reactions to SLS are not common, although a few cases have been reported (20).
- The level of induced irritation can be controlled by adjusting the concentration (21,22).
- Any skin damage is rapidly reversible.

Unlike Other Irritants, Surfactants May Induce Irritation Through Several Pathways

Because of their structure and physicochemical properties, surfactants interact with various targets of the skin: constitutive and functional proteins, intercellular or cell membrane lipids, and living cells.

[a]The term 'anti-irritant' is used to express a reduction of the irritation potential; it does not mean a total suppression of skin irritation.

Table 1 Examples of Potential Skin Irritants

Chemicals
Surfactants (1), solvents (2,3), acids and alkalis (3), dessicants (4), concentrated salt solutions (5), alcohol (6), oils (7), water in wet work conditions (8)
Environmental conditions
Extreme weather conditions [very warm, very cold, dry atmosphere (9), UV radiations or pollution (10)]
Physicals
Abrasives (11), occlusion (12) needles (13), burns (14), rubbing (15)
Biological factors
Some enzymes or combination of enzymes (16), some plants (17)

Table 2 Surfactant-Containing Products

Cosmetics and toiletries
Body-cleansing liquids (shower gels, facial cleansers, liquid soaps, foam baths)
Body-cleansing solids (soap bars, syndet bars, combars)
Shampoos
Shaving products
Toothpastes
Deodorants
Household products
All purpose cleaners
Windows cleaners
Hand dishwashing liquids
Automatic dishwashing products
Fabric detergents
Fabric softeners

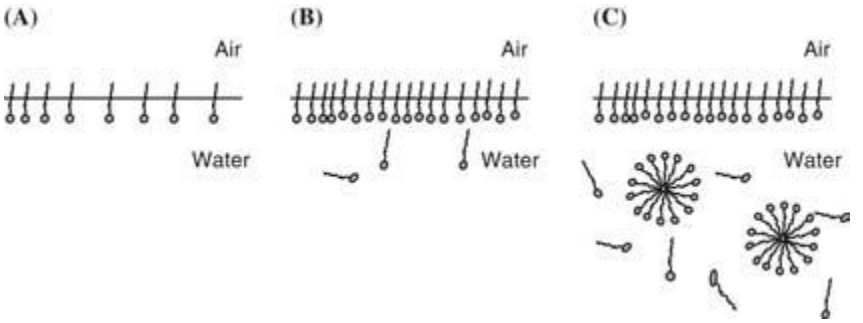

Figure 1 Surfactant behavior in solution. (**A**) Surfactant molecules in aqueous solution concentrate at the air–water interface with the hydrophobic part oriented toward the air side. (**B**) When the concentration of surfactant increases, the interface becomes saturated with surfactant molecules that penetrate into the solution. (**C**) To minimize their interaction with water, the hydrophobic parts of the surfactants interact together and form micelles in solution. These latter are unstable structures that form and disaggregate to establish a balance between monomers and micelles in the solution.

Surfactant Behavior in Solution: Their Physicochemical Properties

Surfactants are amphiphilic molecules, meaning that they contain two opposing parts: hydrophilic (water-loving) and hydrophobic (water-hating). When dissolved in water, the former is readily hydrated while the latter avoids water. As a surfactant is added to water, it concentrates as independent molecules (called monomers) at the air/water interface with the hydrophobic part trying to avoid the water environment. At a certain concentration, called the "critical micellar concentration" (CMC), the surfactant can no longer concentrate at the surface and goes into the bulk of the solution. In order to avoid contact with water, the hydrophobic part of the surfactant molecules tends to aggregate together into larger particles called "micelles" (23) (Fig. 1). However, the hydrophilic part of the surfactant either by repulsive forces between similar charges (for anionic or cationic surfactants) or by trying to interact better with water (all surfactant types) tends to work to disaggregate the micelles. On the basis of those attractive and repulsive forces, micelles are dynamic structures that continuously form and disrupt to define an overall relative proportion

of monomers and of micelles in the bulk. As a consequence, any system that is able to stabilize the micelles or facilitate the incorporation of free monomers into the micelles will reduce the relative proportion of monomers in the solution (24).

MECHANISM OF INTERACTION BETWEEN SURFACTANTS AND THE SKIN

When surfactants come into contact with the skin, they can interact with it in different ways (25):

- By binding to the surface proteins of the skin
- By denaturing skin surface proteins
- By solubilizing or disorganizing the intercellular lipids of the skin
- By penetrating through the epidermal lipid barrier
- By interacting with living cells

All these interactions may lead to irritation. Whatever the mechanism of interaction between the surfactant and the skin, the free monomeric form will be the key driver to initiate irritation as illustrated hereafter.

Interactions with Skin Proteins

Binding of surfactant to isolated stratum corneum (SC), the most external layer of the skin, saturates at or near the CMC (26), which is consistent with the fact that only monomers of surfactants can adsorb to the proteins of the skin (27). After binding to the proteins, surfactants cause the proteins to denature, leading to a swelling of the SC (28). Rhein et al. (29) investigated the swelling of isolated SC when exposed to various single surfactant solutions and showed that the swelling was concentration and time dependent up to the CMC before leveling off. The authors interpreted their results as support for a single interaction between the surfactant monomer and skin proteins.

Denaturation of functional proteins and especially enzymes have multiple consequences such as impaired desquamation process, maturation of lipids and proteins in the epidermis, defense system against free radicals, and enhancement of oxidative stress (30,31).

However, even if the interaction of surfactants with skin surface proteins is related to the CMC of the surfactants or surfactant mixtures, above the CMC there is no more a direct relationship. For surfactants mixtures, it is proposed that above the CMC the affinity of individual surfactants for skin surface proteins also plays a critical role on skin irritation (32).

Interaction with the Intercellular Skin Lipids

The protective lipidic barrier of the skin is composed of highly organized lipid layers located between the cells of the SC. In order to disorganize these lipids and alter the skin barrier function, surfactants have to integrate into the lipidic layers that are mostly hydrophobic. Because of their small size, monomers of surfactants can easily reach the intercellular lipids and disturb the skin barrier function, making such an effect depending on the relative proportion of monomers in solution. However, it has been recently shown (33) that micelles formed from sodium dodecyl sulfate (same as SLS) have a hydrodynamic radius size that is compatible with partial penetration into the SC, and should be capable of interacting with the intercellular lipids. This would partly explain why increasing the concentration of single SLS surfactant solutions above the CMC leads to increased irritation. For other surfactant types, micelles have to release their monomers to interact with the lipidic barrier. The dose-related level of irritation caused by such surfactants above their CMC (34) should thus be related to another mechanism.

Interaction with Living Cells

Once the lipidic barrier has been disrupted or weakened, monomers of surfactants can reach the living part of the epidermis and interact with the keratinocytes and Langerhans cells, leading to the following:

- A lysis of the cells in the case of severe irritants and the release of chemical mediators into the intercellular space

- An alteration of the cellular membrane and passive diffusion of chemical mediators from the cytoplasm into the intercellular space
- A stimulation of the cells with subsequent active release of chemical mediators into the intercellular space or synthesis of new mediators

Whatever the pathway, these mediators will initiate a multitude of reactions at the site of irritation such as a stimulation of cell proliferation, a stimulation of neighboring cells to produce additional mediators, a vasodilatation of blood capillaries in the dermo-epidermal papillae, and an attraction of blood cells. Many different chemical mediators will also be upregulated at the site of irritation such as interleukin (IL)-1 α and β, IL-2, -6, -8, and -10, granulocyte macrophage colony-stimulating factors (GM-CSF), tumor necrosis factor α (TNF-α), interferon-γ, and others. This cascade of chemical messengers responsible for the inflammatory reactions is detailed elsewhere in this book (chap. 42).

Interaction with Neuroreceptors

In sensitive persons, initial contact with some surfactants result in sensory irritation characterized by stinging, itching, or a burning sensation. Such an early signal of irritation was exploited a long time ago with the development of the so-called lactic acid stinging test (35) to detect subjects with an "upper level of skin sensitivity" in the face.

This type of sensory irritation occurs when thin, unmyelinated, chemically sensitive type-C nociceptors are activated and transmit a depolarizing signal via the dorsal root ganglia in the spinal cord to the brain where the sensation is appreciated (36). These receptors are extensively distributed through the dermis and the epidermis allowing excitation, even by faint stimuli. For more intense irritants, a retro-signal is transmitted from the dorsal root ganglia to the inflammation site and contributes to the erythematous reaction.

ANTI-IRRITANTS FOR SURFACTANT-BASED PRODUCTS

Fortunately, nowadays many systems have been developed to minimize the risks of intolerance to cosmetics or surfactant-based products. This is extremely important because of the increased use of toiletry products. They must be as mild as possible to the skin. Not only the mildest ingredients are used, but also finished hygiene products often contain one or more anti-irritant systems.

Anti-Irritation by Using Only Mild Surfactants

The first approach to develop a mild, surfactant-based product is to carefully select the mildest surfactants. Nonionic surfactants are generally considered as the mildest and are typical ingredients in body-cleansing products for babies, for sensitive skin subjects, and for face-cleansing products. However, several anionic surfactants are also extremely respectful of the skin condition and are often introduced in the same categories of products. These are, for instance, highly ethoxylated (at least 5-EO) alkyl sulfates, sulfosuccinate esters, sarcosinates, fatty acid-protein condensate, alkyl phosphate ester, alkyl glutamate, taurates, and others. Amphoteric surfactants are rarely used alone, but rather as secondary surfactant; thus, their intrinsic irritation potential has no real meaning. Cationic surfactants are essentially used for their antibacterial properties rather than their detergent properties and are often described in the literature as the most irritating surfactants. However, like anionic surfactants, it is also possible to find very mild cationic surfactants (e.g., salts of alkyl amine, quaternized alkyl polyglycosides). Because of their low usage, the cationic surfactants will not be discussed in this chapter.

Anti-Irritation by an Appropriate Combination of Surfactants

The best counterirritants for surfactants are other surfactants. Several authors have clearly demonstrated such a positive interaction between various surfactants in vitro (29,37) and in vivo (32,38,39), with diluted (29,37,38) or with highly concentrated solutions (32,39). Amphoteric surfactants are well known to decrease the irritation potential of anionic surfactants (40), but nonionic surfactants can display the same effect when used at a sufficiently high concentration. More surprisingly certain anionic surfactants can reduce the irritation potential of another anionic surfactant, instead of cumulating their effects (39).

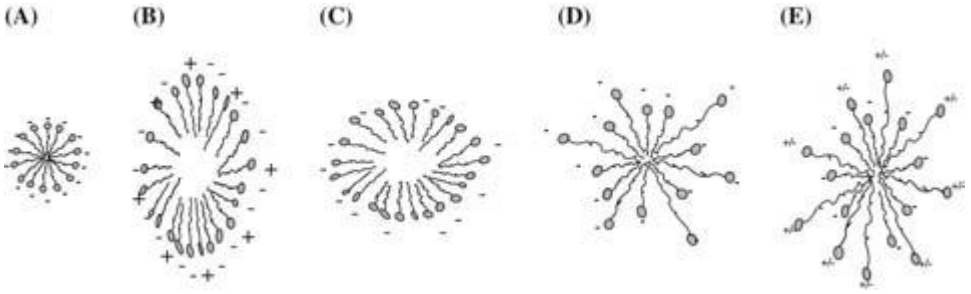

Figure 2 Mixed micelles of surfactants in solution. In aqueous solution, the hydrophobic tail of surfactant monomers form self-assembled aggregates within the core of the micelle, while the hydrophilic head interacts more with the water molecules. This structural arrangement is more energetically favorable because it reduces the unfavorable hydrocarbon/water contact energy. (**A**) When micelles are formed of only one single type of surfactants (e.g., anionic surfactants), electrostatic repulsion forces tend to disrupt the micelles that are not stable. (**B**) Adding cationic surfactants into the micelles increases the size of the micelles, modifies their form, and stabilizes the micelle by introducing attractive charges between the positive and negative polar head groups. (**C**) When adding nonionic surfactants to solution A, uncharged surfactant heads incorporate into the micelles, increasing the size of the micelles as well as the distance between the anionic polar heads. As a result of this increased distance, the repulsive forces between the monomers are reduced, which leads to a stabilization and change of micelle form. (**D**) When a second type of anionic surfactant is incorporated to solution A, the hydrophobic tail should be different from the primary surfactant tail. Consequently, the distance between the anionic surfactant heads is greater, repulsive forces are lower, and the micelles become more stable, are larger and of a different form. (**E**) When amphoteric surfactants are added to the solution of anionic surfactants, their behavior depends on the electronic charge of the surfactant [positive at a pH below the lowest pKa of the surfactant (case B), negative at a pH higher than the highest pKa of the surfactant (case D), and zwitterionic at a pH between the lowest and highest pKa of the surfactant (case E)].

How Can Secondary Surfactants Reduce the Irritation Potential of Primary Surfactants: The Principle of Surfactant Antagonism

Skin is a complex organ with different potential targets for surfactants. Several mechanisms may, thus, occur to explain the reduced irritation observed by mixed surfactant systems as compared with single surfactant solutions.

Overall mechanism for all targets. On the basis of the fact that mainly monomers irritate the skin and that there is an equilibrium in solution between micelles and monomers, any factor able to stabilize the micelles, and hence decrease the relative proportion of monomers, plays a major role in reducing irritation (24). This is the case for secondary surfactants added to the system as explained in Figure 2, but also for other kinds of macromolecules such as proteins or other polymers (41).

As shown in Figure 2, any kind of secondary surfactant is able to stabilize the micelles and reduce the relative amount of irritant monomers in the solution.

Furthermore, in the case of mixed micelles of types B, C, or E (Fig. 2), the overall electrical charge density at the surface of the micelles is lowered. This effect allows the micelles to be less repulsive to surfactant monomers in the surrounding bulk, thus allowing the monomers to more easily incorporate into an ever-expanding micelle. This mechanism explains the reduction in the amount of free monomers in the solution.

Additional mechanism for interactions with surface proteins. The affinity of the monomers for proteins may also displace the monomer–micelle equilibrium. This affinity depends on the intrinsic properties of the surfactant (e.g., ionic charge, tertiary structure, hydrophobic domains, carbon chain length, level of ethoxylation) on the mobility of the monomer in the solution (which is related to the size of the monomer), and on the availability of the protein's binding sites. This latter parameter is mainly significant in the case of concentrated solutions of surfactant mixtures, because their monomers compete for binding sites on the surface proteins as well as with interactions within the micelle. The amount of available monomers that will bind to the protein will thus be decreased, and the irritation potential of the mixture lowered. Such a decrease in the binding of the anionic surfactants to the skin surface has been

demonstrated by attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) in the presence of a secondary surfactant of any type (42).

Similarly, proteins or polymers added to a surfactant solution may also compete for the same binding sites as the surfactant monomers at the surface of the skin and are often counterirritants for the surfactants (42).

Additional mechanism for interactions with intercellular skin lipids. Micelles formed from a single surfactant type are smaller than micelles formed from several surfactant types. While the former category may have a size allowing them to partially penetrate through the SC [e.g., the case for SLS, (33)], the latter examples should be sterically hindered from penetrating into the skin and interacting with the intercellular lipids (43). This effect results in a partial protection of the skin barrier function when adding a secondary surfactant to the primary one.

Scientific Cases of Reduced Irritation in Surfactant Mixtures

Many peer-reviewed scientific publications have reported that mixtures of surfactants are less irritating than expected by the sum of the irritation potential of each species taken separately. Several review papers by Goldemberg (44,45), Effendy and Maibach (46), and Paye (47–49) have illustrated examples of antagonisms between surfactants. The following section focuses on giving additional concrete examples grouped by the type of interaction between the skin and the surfactant investigated by the author.

Interaction of surfactant with proteins in vitro or in vivo

- Ohbu et al. (50) evaluated the protein denaturation properties of surfactants using circular dichroism and demonstrated that the sodium dodecyl sulfate–induced denaturation of bovine serum albumin (BSA) was counteracted by dodecyltrimethylammonium chloride or by *N*, *N*′-dimethyldodecylaminoxide.
- Dominguez et al. (40), using human callus as a skin model, demonstrated a considerable inhibition of adsorption of SLS on the callus when alkyl amido betaine (AAB) was present in the same solution. They deduced from their data that the two individual surfactants were more irritating than any of the combinations tested. They explained their data by a stabilization of the micelles of mixed surfactants and hence a reduction of bioavailable monomers.
- Miyazawa et al. (51) showed in vitro that mixed surfactants reduced protein denaturation compared with single surfactant solutions. Again this was explained on the basis of the reduced level of free surfactant monomers in the mixed surfactant solution as compared with single surfactant solutions.
- Blake-Haskins et al. (52) showed, using an in vitro protein denaturation assay (collagen swelling), that the addition of an amphoteric surfactant to an anionic surfactant reduced the denaturation potential of the anionic surfactant.
- Paye and Jacobs (42), using attenuated total reflection–Fourier transform infrared (ATR-FTIR) spectroscopy, demonstrated, by a study on human volunteers, that the binding of anionic surfactants, SLS, and linear alkyl benzene sulfonate (LAS), to skin surface proteins was significantly reduced when amphoteric or nonionic surfactants were added in the solution. This study illustrated the competition between the two types of monomers for the binding sites on skin surface proteins.
- Tadenuma et al. (53) showed that when alcohol ethoxylate (AE) was added to SLS, the BSA was less denatured. The higher the concentration of AE for a fixed concentration of SLS, the greater the inhibitory effect of AE on SLS-induced protein denaturation. By measuring the adsorption isotherms of SLS onto agarose-immobilized BSA in the presence and absence of AE, the authors correlated reduced protein denaturation by AE with a dramatic reduction of binding of SLS to BSA because of the adsorption of AE onto the protein.
- Paye et al. (54), using commercial surfactants (as provided by manufacturers) and in the exact proportions as in standard commercialized laundry detergents, demonstrated that the protein denaturation potential (using the in vitro zein test) of the mixtures of surfactants was in all cases lower than expected by the cumulative protein denaturation effect measured for the surfactants separately.

Interaction of surfactant with lipids or membranes

- Charaf and Hart (55) investigated in vitro the interaction of surfactants with membranes and demonstrated that the addition of lauryl ether sulfosuccinate to a given concentration of SLS decreased the aggressiveness of the latter surfactant for the membrane.
- Garcia et al. (24) demonstrated that mixtures of surfactants diffused less through a membrane than the same surfactants tested separately. This observation was interpreted by the fact that micelles were too big to penetrate through the membrane and that the relative proportion of monomers was lowered in the mixed solutions.
- Kawasaki et al. (56), using the electron paramagnetic resonance (EPR) technique, demonstrated an increased fluidity of the SC intercellular lipid structure after application of a solution of SLS. This increased disordering was most likely because of an intercalation of SLS monomers into the intercellular lipids organization. The addition of sodium lauryl glutamate (SLG), another anionic surfactant, to SLS inhibited the fluidization of the intercellular lipids caused by SLS alone.
- Moore et al. (43), using dynamic light scattering measurements, determined the size of the hydrodynamic radius for SLS micelles that was compatible with partial penetration inside the SC, while the size of the mixed micelles from SLS and dodecyl hexa(ethylene oxide) (C12E6) was higher and hindered the penetration of the mixed micelles inside the SC. They confirmed their hypothesis by measuring the hydrodynamic radius of surfactants evaluated in skin penetration studies and showed that the addition of C12E6 to the SLS solutions was found to decrease the amount of SLS penetrating into the epidermis. They attributed this decreased penetration to two causes: a decrease in the concentration of SLS monomers because of a stabilization of the micelles and a decreased penetration of the mixed micelles because of an increase in their steric size.

Interaction of surfactants with living cells in vitro

- Earl et al. (57) showed in a three-dimensional cell culture model of human skin that equal mixtures of SLS and *N*,*N*-dimethyl-*N*-dodecylaminobetaine have reduced cytotoxicity potential compared with their single applications at the same concentrations. Their observation correlated well with the results of a four-hour human patch study in which the same single surfactants were tested versus the surfactant combinations at high concentrations.
- Benassi et al. (58), using cell culture models, demonstrated that the cytotoxic effect of SLS was reduced when it associated with different tensides such as cocamidopropyl betaine, polysorbate-20, and polysorbate-80. They compared their results to previous data showing that the barrier damage caused by SLS in vivo was lower when SLS was used in combination with other tensides because they were able to reduce the CMC of SLS.

Interaction of diluted surfactant solutions with skin in vivo

- Rhein et al. (59) used in vivo skin irritation studies (21-day cumulative irritation test) to show that the addition of (C12-C14) alkyl, 7-ethoxy sulfate (AEOS-7EO) to a constant dose of SLS resulted in a significant reduction of erythema, hence producing a milder system.
- Marti (60), using four in vitro and two in vivo models for skin and mucous membrane irritation prediction, showed that the irritation potential of sodium lauryl ether sulfate used as a primary surfactant could be significantly decreased by adding cocamidopropyl betaine, or coco amphocarboxypropylate, or protein fatty acid condensate as secondary tensides for mildness synergy in shampoo formulations.
- Zenhder et al. (61) evaluated the effect of sodium laureth carboxylate with two different levels of ethoxylation (5 and 13 ethoxylations) for their effectiveness in reducing the irritation potential of SLS in a five-day human patch test. Both carboxylates were counterirritants to SLS as shown by clinical examinations, measurements of superficial blood flow, skin barrier alterations, and skin electrical conductance.

- Lee et al. (38), in a 24-hour patch test, showed that adding SLG to a solution of SLS decreased the irritation potential of the latter.
- Teglia and Secchi (62), using a three-week arm-soaking test on human volunteers, showed that the amphoteric surfactant, cocamidopropyl betaine, had a similar anti-irritant effect versus wheat protein when added to a solution of SLS. Both the wheat protein and cocamidopropyl betaine protected the skin against alteration of the skin barrier and subsequent irritation.
- Teglia and Secchi (41) reported that using SLS, sodium laureth sulfate (SLES), and olefin sulfonate as primary surfactants, and obtaining some new formulations by mixing these primary surfactants with four different auxiliary surfactants and protein hydrolyzates, reduced the damage to the SC.
- Paye and Cartiaux (63) showed in a short-term patch test on human volunteers that alkyl betaine (amphoteric surfactant) and AE (nonionic surfactant) reduced the alteration of the SC caused by SLS or by LAS (anionic surfactants).
- McFadden et al. (64) mentioned in one of their publications that they had run an unpublished clinical study demonstrating that the direct addition of benzalkonium chloride (BC) to a solution of SLS reduced the irritant inflammatory response of the volunteers to SLS. They explained their observation by a stabilization of the mixed micelles by BC.
- Vilaplana et al. (65) emphasized the importance of the physicochemical behavior of surfactants in solution as a way of minimizing their irritant properties. In a 48-hour patch test on human volunteers, the authors showed that the addition of disodium cocoyl glutamate or of sodium PEG-4 lauramide carboxylate to a solution of SLS produced a significant reduction in the transepidermal water loss (TEWL), skin color reflectance, and laser Doppler velocimetry, even though there was a two-time increase in the total surfactant concentration.

Interaction of concentrated surfactant preparations with skin in vivo

- Dillarstone and Paye (39), using the four-hour human patch test with concentrated surfactant systems, demonstrated that the addition of 10% of the following surfactants: cocoamidopropylbetaine, ethanolamide, SLES, or AE to a solution of 20% SLS, or of LAS decreased in all cases the level of erythema induced by the anionic surfactant alone, even though the overall concentration of surfactant in the mixture was increased. Even more, a solution with 20% LAS + 10% SLES + 10% AE (total concentration of 40%) was found to be less irritating than a solution of only 20% LAS.
- Hall-Manning et al. (32), using the four-hour human patch test, investigated the interaction between highly concentrated anionic and amphoteric surfactants and showed that the irritant effect on skin of the mixtures (20% of sodium dodecyl sulfate + 20% of dimethyl dodecyl amido betaine) was significantly lower than the effect of the anionic surfactant (at 20%) tested alone. The authors pointed out the correlation between the reduced irritation and the reduced CMC for the mixture of surfactants. However, at such a high surfactant concentration, they also attributed the lowered irritation potential to a reduced affinity of the individual surfactants for the skin proteins.

Anti-Irritation by Polymers or Proteins/Peptides

The counterirritant capability of polymers or proteins on surfactants has been reported in the literature (61,66–68). The mechanism by which polymers and proteins function is similar to the one described above for surfactant mixtures. They incorporate into the micelles and thus decrease the relative amount of free monomers in solution. Their skin substantivity can also involve blocking binding sites at the surface of the skin, thus making them nonaccessible to surfactants.

Polymers or proteins differ in their ability to interact with the skin surface and to be incorporated into the micelles. The following parameters should be considered when selecting a polymer/protein:

- Better interaction with the micelles correlates with increased hydrophobicity (66).
- Better substantivity to the skin correlates with higher hydrophobicity when the polymer is quaternized or is cationic or when the net charge or the size of the polymer/protein increases (67,68).

As stated above, more hydrophobic and/or larger polymers/proteins are much more effective to depress the skin irritation potential of surfactants.

However, in the literature, the anti-irritant effect of proteins/polymers in the presence of surfactants has been demonstrated mostly in single surfactant solutions, and at a high polymer-surfactant ratio, not always compatible with other properties of the finished product. When they are formulated into finished products already optimized for skin compatibility through an appropriate combination of surfactants, most polymers or proteins do not bring any further mildness benefit to the product (unpublished data).

Anti-Irritation by Refattening Agents

One of the negative effects of surfactants on skin is the alteration of its lipid barrier. This can be easily assessed by measuring the TEWL (56,69), which increases with an impairment of the barrier. Using refattening ingredients or skin barrier–repairing ingredients in surfactant-based products can reduce the disruption of the barrier function if those ingredients are appropriately delivered to the skin surface. Such ingredients are often the basis for the barrier cream effect of creams (lotions) topically applied before or after contact with an irritant. Some of these ingredients can also be formulated into a surfactant system and act directly as anti-irritants in the mixture. The occlusive effect they bring at the surface of the skin delays the water loss and maintains the skin in a less-dehydrated state. Furthermore, they can progressively form an additional barrier protecting the skin against the surfactants in repetitive product applications conditions. Several types of refattening ingredients are available and can be formulated in surfactant systems. Among these are ethoxylated mono-, di-, and tri-glycerides, fatty alcohols and ethoxylated fatty alcohols, fatty acid esters, lanolin derivatives, or silicone derivatives. A few products containing a high percentage of oil also exist and can possibly be added to surfactant systems to serve in a barrier protection role.

Anti-Inflammatory Effect

Anti-inflammatory ingredients are not specific for surfactant-induced irritation, and most of them are used in pharmacology rather than in cosmetology. Because of the complexity of the inflammatory process, several families of anti-inflammatory ingredients have been developed such as glucocorticoids, nonsteroidal anti-inflammatory drugs (tacrolimus, cyclosporin, rapamycin, ascomycin, and leflunomide), flavonoids, essential oils, or α-bisabolol (70–72). In order to be effective, such ingredients must be delivered to the skin in a bioavailable form and in a sufficient amount. The case of essential oils, flavonoids, and α-bisabolol is discussed in more details in other sections of this handbook.

Antioxidants

In biological systems, antioxidants processes have a protective role against oxidative stress through three different mechanisms:

- By scavenging the early pro-oxidant species
- By preventing the initiation or the propagation of the free-radical reactions
- By returning oxidized groups to their reduced state

In dermatology and cosmetology applications, antioxidants belong to a relatively new field of investigation and interest. Some of the most important antioxidants with known applications are vitamin E, vitamin C, thiols, polyphenols, and flavonoids. Their mechanism of action in the antioxidant process is reviewed by Weber et al. [Chapter 28]. In surfactant-based products, antioxidants are only occasionally used to reduce the skin irritation potential of the product (73). However, several cutaneous enzymes are involved in the protection of the skin

against free radicals and reactive oxygen species (ROS). Such enzymes are partly denatured once surfactants penetrate the skin and the natural defense mechanisms of the skin may then become overwhelmed leading to an oxidative stress situation. Any supplementation of the skin with scavenging systems to, for example, combat surfactant irritation could result in a reduced irritation response.

Anti-Sensory Irritation

Although much less discussed than the clinical irritation, which is characterized by observable or functional alterations, subjective irritation also exists. It does not have great interest for the dermatologists, but for cosmetologists it can be the reason consumers like or reject their product.

Three different categories of sensory signals of irritation have been identified. Briefly, these are

- stinging, burning, and itching signals;
- dryness and tightness perception preceding clinical signs of irritation; and
- peculiar "irritated skin"–perceived signals unrelated with a true irritation process.

These types of irritant signals will require different "anti-irritant" systems.

Anti-Irritants for Stinging, Burning, and Itching Sensations

Strontium salts have been demonstrated to be effective and selective anti-irritants for chemically induced sensory irritation associated with stinging, burning, or itching manifestations (36). Strontium salts (nitrate or chloride) are claimed to be especially indicated for subjects with sensitive facial skin and prone to stinging sensations (36,74). The interest of strontium salts, as described by Hahn (36), is that they are very specific and selective inhibitors of the sensory signals of irritation, without suppressing other receptors (such as temperature, tactile, pressure, etc.).

Several controlled clinical studies (36,75) were run to show that strontium nitrate or chloride, at concentrations from 5% to 20%, effectively suppressed or reduced sensory irritation caused by chemical or biological irritants over a wide range of pHs from 0.6 to 12. In tests, the strontium salts were included in the solution with the irritant, or before or after the application of the irritant as shown in Table 3.

Although not tested in surfactant systems, strontium salts may play a similar beneficial effect on surfactant-induced sensory irritation, mainly in certain classes of sensitive skin subjects.

As described within the mechanism of sensory irritation, it has been observed in some studies that, on top of reducing the sensorial signs of irritation, strontium salts could also decrease the level of erythematous reactions generated by the irritant.

Table 3 Clinical Tests Support the Anti-Sensory Irritant Potential of Strontium Salts

Irritant	Test site	Timing of application[a]
Lactic acid, 7.5%, pH 1.9 (solution)	Face	Mixed, pre or post
Lactic acid, 15%, pH 3.0 (solution)	Face	Mixed
Glycolic acid, 70%, pH 0.6 (peeling solution)	Arm	Mixed
Capryloyl salicylic acid, 1% (exfoliant cream)	Cheek	Mixed
Ascorbic acid, 30%, pH 1.7 (solution)	Face	Mixed
Aluminum chloride, 20% (antiperspirant preparation)	Axilla	Pre
Aluminum/zirconium salt, 25% (antiperspirant solution)	Arm	Mixed
Calcium thioglycolate, pH 9–12 (depilatory lotion)	Leg	Post
Histamine (intradermal injection, 100 μg)	Forearm	Pre

[a]"Pre" means that strontium salts were applied to skin prior to the irritant, "post" means that the salts were applied after skin had been irritated by the irritant, and "mixed" means that strontium salts were included in the preparation with the irritant.

Source: From Ref. 36.

Although several hypotheses have been communicated to explain the mechanism of action of strontium salts (36,76), the mode of action still remains unclear. Below, please find some of author's thoughts on this matter:

- Because strontium salts mitigate the irritant event immediately after application, it is assumed that they act directly on the type-C nociceptor and suppress the neuronal depolarization that normally transmits the sensory signal to the brain.
- By their analogy to calcium, strontium salts could also use calcium channels to induce the release of neurotransmitters in synapsis or could antagonize the usual calcium-induced depolarization.
- It is also not impossible that strontium salts could directly influence keratinocytes or inflammatory cells and regulate the release of some cytokines.

Anti-Irritants for Dryness/Tightness Perception

Tightness and dryness perception are usually the earliest warning signs detected by highly receptive subjects using products that are not irritating with one single use but can become slightly irritating or skin drying after multiple exposures. These signs are generally followed, if the product is not discontinued, by the progressive development of clinical signs of intolerance such as scaling, flaking, or even erythema (77).

This kind of subclinical irritation is essentially observed for surfactant-based products and refattening agents, as described above, should be incorporated into the formula at a high concentration to mitigate the drying effect. Additionally, topical skin rehydrating preparations can also be effective in some cases to decrease the dryness/tightness perception.

Anti-Irritants for Negative Sensory Skin Feel

Negative subjective sensory signals that are translated as "irritated skin" by the consumers while totally independent of irritation can be addressed in two ways:

- If these signals are induced by the surfactant-based product, the surfactant system should be reformulated. Indeed, each surfactant is associated with a specific perception to the skin such as slipperiness, smoothness (perception of a mild product) or, at the extreme, roughness, and drag (perception of an irritant product). A good combination of surfactants can provide the desired skin feel and signal.
- Skin feel additives may be added to the product to deliver smoothness, silkiness, and a hydrated feel associated with a "non-irritated" skin signal. A review of the skin feel additives has been made by Zocchi in another section of this handbook (Chapter 34).

The Effect of Divalent Cations on Skin Irritation

Magnesium is not an Anti-Irritant for Surfactants (78)

Magnesium is frequently described as a depressor of skin irritation. Such a false idea is essentially arising from in vitro data based on protein denaturation tests. In those tests, the more a surfactant solution denatures a protein, the more it is predicted to be an irritant to the skin; and magnesium clearly depresses surfactant-induced protein denaturation in vitro (79). However, when well-controlled in vivo tests were performed to investigate the effect of magnesium directly on human volunteers, it was confirmed that magnesium does not decrease the skin irritation potential of surfactants or surfactant-based products (78). The in vivo studies included both acute irritation by occlusive patch tests and chronic irritation by repetitive short-term applications of the products. The study compared sodium and magnesium salts of surfactants (e.g., magnesium and SLS) in single solutions or incorporated into finished products and investigated the effect of adding magnesium sulfate to a solution of surfactant.

Some preliminary studies with calcium showed a behavior similar to magnesium (personal data) with an inhibition of protein denaturation in vitro while no reduction of irritation in vivo.

Zinc Salts May be Potent Anti-Irritants for Surfactants

Zinc is a key co-element in more than 200 enzymatic reactions that happen in the skin and is, as such, of critical importance to the skin (80). A few publications have shown the beneficial

protective effect of topically applied zinc oxide on skin irritation (81) and as a mediator of oxidative stress (82). Zinc oxide has also been incorporated into skin protective aerosol compositions to protect a baby's bottom from erythema (83), and in surfactant-based liquid products to exert a substantial anti-irritant effect on skin (84). Other zinc salts have been incorporated in leave-on products (gels, creams, lotions, or ointments) to reduce or prevent dermal or mucosal irritation (85).

More recently, Rigano L et al. have shown that zinc salts of coceth sulfate were very mild to the skin and that zinc salts of lauryl ether sulfate were milder than their sodium counterpart (86).

In view of the many situations in skin where zinc plays an essential role, the exact mechanism by which the zinc element exerts its beneficial effect on skin irritation has still, however, not been elucidated.

CONCLUSION

This chapter describes how surfactants interact with the skin and briefly reviews several systems by which it is now possible to control the skin irritation potential of surfactant-based products. This can be done through a

- modification of surfactant behavior in solution;
- modification of surfactant interaction with the skin surface;
- protection of the skin surface via ingredients (e.g., lipids, proteins, and polymers); and
- control of their subjective perception by the consumer using strontium salts or skin feel agents.

These anti-irritant systems, combined with a selection of mild surfactants allow the cosmetic formulator to design very mild hygiene products.

Other anti-irritant systems also exist for leave-on cosmetics and in pharmacology such as antioxidants and anti-inflammatory ingredients. They are still not yet commonly used in surfactant-based products but, if correctly delivered to the skin during the use of the product, they could provide a new field of research for improving the tolerance of cleansing products.

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44 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 (Tabel 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 (EU), 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 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 the features of inflammation to the desired context. Therefore, predicting human responses on the basis of 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 is described

Table 1 Classification of Irritant Dermatitis

Classification	Features	Clinical picture
Acute irritant dermatitis	Single exposure	Reaction usually restricted to exposed area, appears within minutes
	Strong irritant	Erythema, edema, blisters, bullae, pustules, later eschar formation
	Individual predisposition considered generally unimportant	Symptoms include burning, stinging, and pain
		Possible secondary infection
		Good prognosis
Irritant reaction	Follows repeated acute skin irritation	Repeated irritant reactions may develop into contact dermatitis
	Often occupational; hairdressers, wet workers	Good prognosis
Cumulative irritant dermatitis	Repeated exposures required	Initially subject may experience stinging
	Initial exposures cause invisible damage	Eventually erythema, edema, or scaling appears
	Exposure may be weeks, months, or years until dermatitis develops	Variable prognosis
	Individual variation is seen	
Delayed acute irritant contact dermatitis	Latent period of 12–24 hr between exposure and dermatitis	Clinically similar to acute irritant dermatitis
		Good prognosis
Subclinical irritation	Irritation detectable by bioengineering methods prior to development the 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
Pusstular 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

Table 2 Draize-FHSA Model

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

Abbreviation: FHSA, Federal Hazardous Substance Act.

in Tables 2 and 3 (3–5). Table 4 compares this method with some other modifications of the Draize model.

Draize used a 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 non-abraded). 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

Table 3 Draize-FHSA Scoring System

	Source
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	4
Formation (injuries in depth)	
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

Abbreviation: FHSA, Federal Hazardous Substance Act.
Source: From Ref. 4.

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	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 (hr)	24	24	4	4	4
Examination (hr)	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 S2 or > 11.5	Toxic materials pH S2 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: From Ref. 4.

irritating. A value of 5 defines an irritant by Consumer Product Safety Commission standards. Subsequent laboratory and clinical experience that has shown the value judgments (i.e., nonirritating, mildly irritating, 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.

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 over-predicting 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 two weeks to shaved back skin of young guinea pigs. Barrier creams were applied to the test animals two hours before and immediately after exposure to the irritant. Control animals were only treated with the irritant. Erythema was measured visually, and by bioengineering methods: laser Doppler flowmetry and transepidermal water loss (TEWL). 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.

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 three-week period, and the results are measured with a visual score for erythema and skin thickness measurements. These two parameters are highly correlated. 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 three applications per day for three 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 immersed in a 40°C solution for four 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 (13–16). 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 were 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 with the mechanism of capsaicin-induced inflammation. Mice were pretreated with various receptor antagonists, such as 5-HT2, HI, 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 non-occlusive 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 four-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 (Tabel 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 four-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 by 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 four-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 in^2 of Webril® was saturated with a 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 was removed after 24 hours, and then reapplied after examination of the test site. This was repeated for 21 days and the IT50 could 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 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, TEWL, and chromametry.

In the first method, MWF were applied with Finn Chambers® on the volunteers' mid-back, removed after one day of exposure, and reapplied further for two days. In the second method, cumulative irritant contact dermatitis was induced using a repetitive irritation test for two weeks (omitting weekends) for six hours per day. The three-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., TEWL) 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 were perpendicular to these. Duhring chambers, containing 0.1 g of test materials (ointments, creams, or powders), were then placed over the test sites. For liquids, a saturated fitted pad (0.1 mL) may be used. Chambers containing fresh materials are reapplied daily for three 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 skins are tested to reflect the relative degree of irritation between compromised and intact skins; 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 and 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 six hours. The skin is examined daily before patch application and on day 8, the final study day. No patches are applied after day 5. Applications 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 to improve irritancy prediction by mimicking consumer use. Kooyman and 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 five 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 and hand immersion assays were developed (5).

Clarys et al. (35) used a 30-minute/four-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 assessing the stratum corneum (SC) barrier function (TEWL), 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 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 the 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 pre-treated 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 non-atopic medical students, each subject washed the outer aspect of the one forearm with liquid detergent for one minute, twice daily for one 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: TEWL, electrical capacitance, and skin blood flow. In the wash test, atopies and non-atopics developed irritant contact dermatitis equally, whereas atopies 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, TEWL, 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 nonparametric data. Of the barrier creams studied, paraffin wax in cetyl alcohol was found to be the most effective in preventing irritation.

Wigger-Alberti and Eisner (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 TEWL 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. (7) 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 two weeks. One arm of each subject was pre-treated with a barrier cream. As in the animal model, erythema was assessed by visual scoring, laser Doppler flow, and TEWL. Skin color was also measured by colorimetry (La^* value). The barrier cream decreased skin irritation to SLS, the most differentiating parameter being TEWL 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 skill in investigations, subjective assessment of erythema, edema, and other visual parameters may lead to confusion by inter-and intra-observer 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 TEWL, capacitance, ultrasound, laser Doppler flowmetry, spectroscopy, and chromametric analysis, in addition to skilled observation may increase the precision of the test. Andersen and Maibach (42) 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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45 Noninvasive Clinical Assessment of Skin Irritation/Inflammation

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INTRODUCTION

The study of skin biology has been undergoing a transformation over the past 20 years, owing to advances in technology that permit evaluation of parameters invisible to the naked eye. Many years ago, Kligman coined the term "invisible dermatoses" to emphasize that what appears visually normal can be quite abnormal under the skin surface (1). He even suggested that the future of dermatology would become so reliant on non-visual methods of diagnosis that the inability to see would not preclude one from a career in the field (2). Evolving techniques and instrumentation have facilitated the study of many of the skin's physiological and biophysical properties, including water content, barrier properties, tensile strength, and elasticity, and even estimates of melanin, hemoglobin, and collagen. For those interested in the immune and inflammatory response of the skin, however, instrumental methods have been less useful.

Surface assessment and grading of inflammatory skin reactions have long relied on visual methods (3–5). Certain instrumental methods, such as laser doppler flowmetry and colorimetry, provide some degree of numerical quantification of surface skin reactions that can supplement simple visual grades (6). However, the detailed study of the cells and molecular processes underlying skin inflammation has generally required use of highly invasive (e.g., biopsy), or moderately invasive (e.g., suction blister), techniques (7–10).

In the mid-1990s, new techniques were introduced to study skin inflammation by attempting to adsorb molecular mediators of inflammation from the skin surface or within layers of the stratum corneum. First introduced in the mid-1990s by Japanese investigators, the approach was to use a relatively mild cellophane tape-stripping method (11) and measure extracted constitutive cytokine levels (and ratios) in sun-exposed, unexposed, and UV-irradiated skin. At the time this work first appeared in print, our laboratory was independently developing and later presented (12,13) and published (14) an even less-invasive approach that used minimally adhesive Sebutape® for mediator adsorption. Over the next several years, both groups reported on the additional application of these procedures for the "targeted proteomic" detection of inflammatory mediators (cytokines) in various types of compromised skin conditions and diseases. Other investigators have more recently adopted these techniques, using somewhat more aggressive tape-stripping techniques to probe cytokine or mRNA expression patterns at deeper levels of stratum corneum.

CELLOPHANE TAPE ADSORPTION OF PROTEIN MEDIATORS OF SKIN INFLAMMATION

The original procedure for noninvasive tape adsorption of human skin for assessment of inflammatory mediators was that of Hirao and coworkers, who used cellophane tape to extract the constitutive cytokines interleukin-1 alpha (IL-1α) and its competitive inhibitor interleukin-1 receptor antagonist (IL-1ra) from the stratum corneum of sun (UV)-exposed and unexposed skin (11). Their basic procedure was to cleanse the sampling site with soap and water, tape strip once with cellophane tape (which was discarded), and re-strip the same site. The second tape was extracted (sonicated in buffer) and assayed for immunoreactive IL-1α and IL-1ra. The cytokines were measured directly by enzyme immunoassay as well as by immunoblotting and functional (induced cell proliferations) assays.

In comparing sun-exposed (face) and unexposed (inner arm) skin sites, they saw higher levels of IL-1α on the arm versus face and higher levels of IL-1ra on the face. Levels of both cytokines were normalized to total recovered protein. The ratio of IL-1ra/IL-1α was approximately 8 on the arm and >100 on the face. IL-1ra activity and the IL-1ra/IL-1α ratio were also increased on UV-unexposed back skin for one to four weeks after 2 MED irradiation of the skin. These results suggested that chronic inflammation due to UV from either natural sun exposure or UV lamp irradiation was associated with elevated IL-1ra production, perhaps a regulatory response to IL-1α-induced inflammation and an effort by the skin to quell this response and restore homeostatic balance.

Two years later, this same laboratory expanded on their initial findings by again demonstrating increased ratios of IL-1ra/IL-α in inflamed skin (15). In this second study, they examined involved versus uninvolved skin sites in subjects with psoriasis, atopic dermatitis, and senile xerosis. Their interest in psoriasis stemmed from earlier findings from Cooper's laboratory (using keratomed epidermal skin samples) that involved psoriatic skin had increased IL-1ra/IL-1α ratios compared with uninvolved skin from the same patients (16). The tape-stripping procedure they employed in the second study was a slight variation of the original method insofar as only a single tape application was used.

They confirmed increased IL-1ra/IL-1α ratios in sun-exposed versus unexposed skin and also observed increased ratios in all of the inflammatory skin conditions versus uninvolved skin from the same subjects. Because of intersubject variability in the measured cytokine amounts recovered, not all the comparisons were statistically significant; however, there were obvious directional changes even for those comparisons that were not significantly different. Their conclusion from these studies was that an increased IL-1ra/IL-1α ratio in the stratum corneum represents a nonspecific phenomenon in any inflammatory skin condition; likely reflecting a regulatory response against unchecked inflammation.

SEBUTAPE ADSORPTION OF PROTEIN MEDIATORS OF SKIN INFLAMMATION

The work of Hirao et al. was unknown to us as we began to investigate the possibility of using noninvasive tape adsorption methods to assess mediators of skin inflammation back in late 1994. We examined a variety of approaches to this problem, including a variety of tapes, extraction methods, and mediators of interest. We settled upon Sebutape as the adsorbent tape of choice for two main reasons. An acrylic polymer film manufactured by CuDerm, this tape was much less adhesive and adherent to skin than either cellophane tape or another CuDerm tape product, D-Squame® (a polyacrylate ester adhesive). Multiple one-minute applications of Sebutape to the same skin site did not strip off the stratum corneum, unlike the other two tapes. As noted in our first publications on the method, we obtained complete and quantitative recovery of spiked cytokine when the Sebutapes were sonicated and vortexed to extract the material (12,14).

Sebutape turned out to be a very flexible adsorbent tape for our purposes. It could be applied to overtly inflamed skin (including infant skin) without causing pain on removal. It could also be applied to mucosal surfaces and trimmed and applied to tight spaces with limited skin surface area (e.g., scalp skin between parted hairs). Proteins (cytokines) of interest were assayed by enzyme immunoassay and recoveries were normalized to total protein to reduce intersubject variability. Figure 1 shows some of the Sebutape application methods used in our studies.

Reapplication of up to 30 of these tapes to the same area of skin did not produce any erythema—common with cellophane tape stripping—and also tended to recover similar amounts of the cytokine IL-1α (Fig. 2) with each application, confirming historical findings that the stratum corneum acts as a reservoir (sink) for this cytokine (18). No inducible cytokine (IL-8) was detected even 24 hours after the initial tape collections—again indicating a lack of tape-induced irritation.

Consistent with the work of Hirao and coworkers, we found a reproducible elevation of IL-1ra and the IL-1ra/IL-1α ratio on sites of the body (face, lower leg, forearm) that were prone to sun exposure and lower ratios on generally sun-protected skin sites (upper leg, back, underarm) (Fig. 3). We also observed elevated IL-1ra/IL-1α ratios and evidence of induced IL-8 (Fig. 4) on infant skin associated with different types of diaper dermatitis. In contrast, an

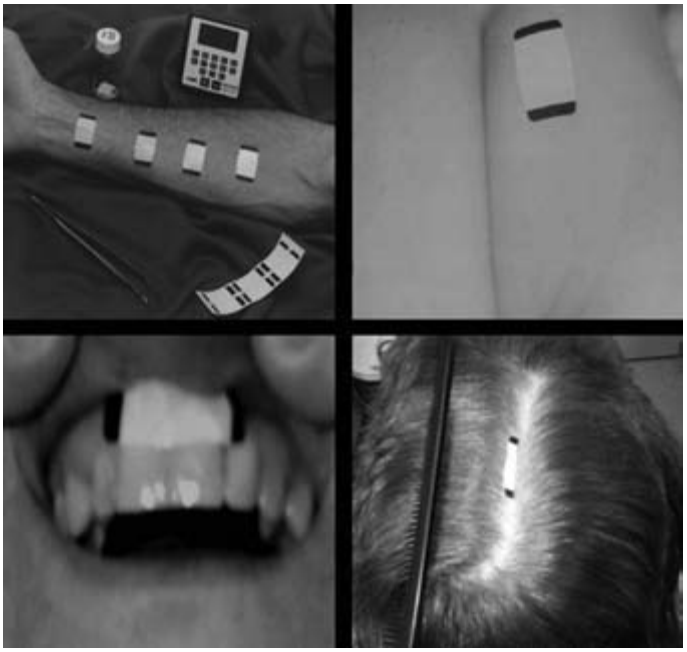

Figure 1 Photographs of Sebutape® application to various skin and mucosal surfaces. *Source*: From Ref. 17.

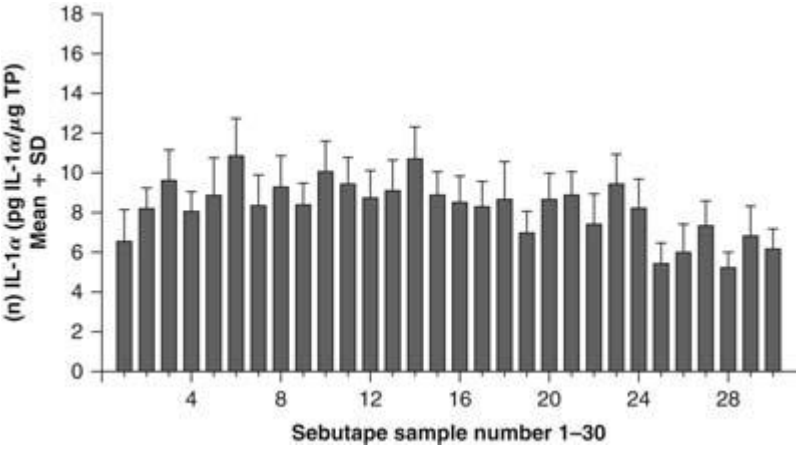

Figure 2 Recovery of IL-1α from Sebutape® samples collected from the same forearm skin site. Thirty successive Sebutape samples were collected from the same normal-appearing naive skin site on the lower volar forearm of four subjects ($n = 2$ sites/subject). Each bar represents the group mean IL-1α level (±SD; $n = 8$ samples) for each of the 30 Sebutape™ samples collected from each of the two skin sites. The IL-1α/total protein levels from samples 1 to 30 range between 5 and 15 pg IL-1α/mg total protein. There were no detectable levels of the inducible cytokine IL-8 (assay sensitivity 10 pg/ml) in any of the tape extracts, including samples collected 24 hours after the initial tape collection. *Source*: From Ref. 14.

acute (1 hour) exposure to a high concentration of the irritant surfactant, sodium dodecyl sulfate (SLS), which is sufficient to produce a weak erythematous response 24 hours after exposure (5), produced an opposite effect. In this situation, IL-1α (measured 24 hours after the SLS exposure) was elevated and the IL-1ra/IL-1α ratio was decreased. This indicated that IL-1α is induced and mobilized in the acute irritation response and that the IL-1ra levels increase later on; again supporting the role of elevated IL-1ra as a means to regulate more chronic inflammatory responses.

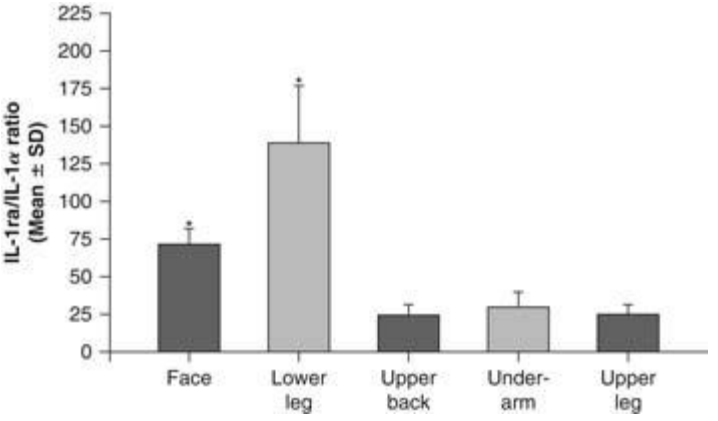

Figure 3 Cytokine levels in sun-exposed versus unexposed skin. Sebutape® samples were collected from different body sites of adult (aged 18–65 years) male and female subjects with normal-appearing unblemished skin. The ratio of IL-1ra/IL-1α for sun-exposed facial skin (mean $\pm$ SD of all sites) and lower leg were significantly higher ($\sim$3–6 times, respectively) than skin that was minimally sun-exposed (upper back, underarm, upper leg). *Source*: From Ref. 14.

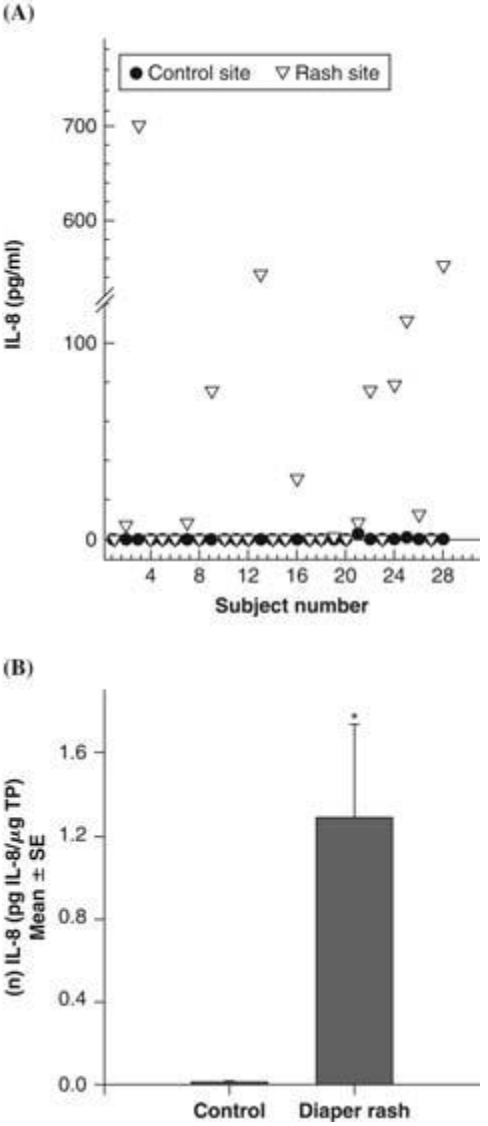

Figure 4 Correlation between skin reactions in diaper area and IL-8 recovery. Sebutape® samples were collected from infant skin sites with different rash severity and from control leg sites. Individual levels of IL-8 for each subject ($n = 28$) for control and diaper rash sites (all rash grades) are shown (**A**). The normalized IL-8 levels (**B**) were significantly higher in rash versus control sites (* $p \leq 0.05$; paired Student's t-test). *Source*: From Ref. 14.

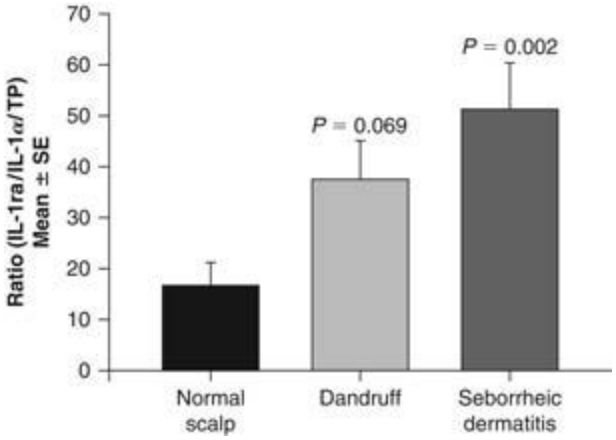

Figure 5 The ratio of IL-1ra/IL-1α normalized to total protein was significantly increased in the seborrheic dermatitis scalp group compared with normal scalp controls. The dandruff group approached significance when compared with the normal scalp group. *Source*: From Ref. 19.

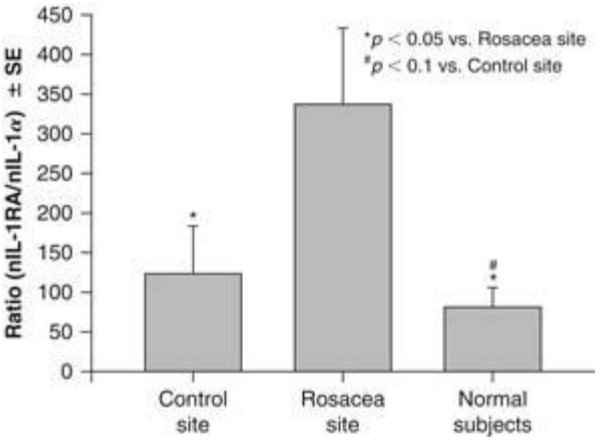

Figure 6 The ratios of normalized interleukin-1ra/interleukin-1α were determined for rosacea subjects (involved and noninvolved control sites) and normal subjects. *Source*: From Ref. 22.

The elevation of the ratio of IL-1ra/IL-1α was a hallmark indicator of every inflammatory condition (skin or mucosal) that we studied over a period of six years. In addition to our findings with sun-exposed skin and infant diaper dermatitis noted above, we saw directional or significant elevation in this ratio in dandruff and seborrheic scalp dermatitis (19) (Fig. 5). Seborrheic dermatitis and dandruff were also associated with elevated recovery of the inducible immune or inflammatory cytokines IL-2 and TNF-α, respectively. Rosacea, an inflammatory skin condition (20), with known cytokine involvement (21,22) was also shown to be associated with elevated IL-1ra/IL-1α ratios (23) (Fig. 6). Involved skin sites showed elevated ratios compared with uninvolved skin sites from the same subjects. However, even the uninvolved sites showed slightly elevated ratios compared with facial skin sites sampled from normal control subjects.

In addition to our work on skin and overt inflammatory conditions, we expanded this technique to other realms of the inflammatory response. A common technique for the study of oral inflammation (e.g, gingivitis) is to try to recover small amounts of gingival fluid for assay. As shown in Figure 1, we were able to apply Sebutape over the gum surface and adsorb cytokines as an alternative, a somewhat simpler method (24). The objective of the study was to examine the relationship between changes in cytokine levels and clinical inflammation. Subjects participated in a 14-day experimental gingivitis (EG) model, where five days following a dental prophylaxis subjects refrained from all oral hygiene measures for 14 days. A gingivitis index (GI) and gingival bleeding were assessed clinically by standard techniques. Sebutape samples of each subject's gingival surface were collected from the right posterior buccal quadrant at baseline (pre-EG) and day 14 (post-EG). The tapes were analyzed for both IL-1α and IL-1β. Over a 14-day EG period, statistically significant ($p < 0.05$) increases in GI, gingival bleeding, and IL-1α (Fig. 7) were observed. A directional increase in IL-1β was also observed.

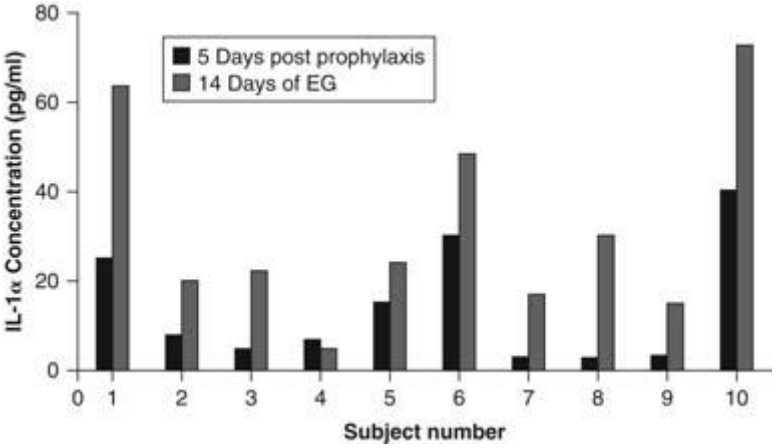

Figure 7 Sebutape samples were collected from 10 adult clinical subjects in an experimental gingivitis (EG) model. Baseline samples were collected 5 days after dental prophylaxis (*black bar*) and then after 14 days of EG (*gray bar*). Samples were extracted in saline and analyzed for IL-1α.

A final adaptation of this method was to try and identify skin surface biomarkers that could be associated with, and diagnostic of, neurosensory skin irritation. Because sensory irritation is purely symptomatic in nature, we applied this technique to see if a more objective index of the response could be developed through the Sebutape biomarker adsorption method. Though we were unable to completely investigate this hypothesis, we did have some early success in demonstrating reduced nitric oxide (NO) recoveries from the skin of test subjects experiencing weak-to-moderate stinging responses to lactic acid or capsaicin (25). Looking at cytokine recoveries, we also saw reduced IL-1ra levels in dandruff subjects after one and two weeks use of an antidandruff shampoos versus a placebo shampoo. This reduction was seen in parallel with a reduction in scalp itch symptoms (unpublished). Assuming that the antidandruff shampoo active was effective in helping to quell the inflammatory response in the scalp, this reduction in IL-1ra is consistent with its role in downmodulating the Il-1α triggered inflammatory response. A reduction in inflammation due to shampoo treatment would likely result in lower endogenous IL-1α and a resultant reduction in IL-1ra.

RECENT ADAPTATIONS OF SKIN TAPE APPLICATION METHODS FOR ASSESSMENT OF STRUCTURAL AND INFLAMMATORY PROTEINS AND GENE EXPRESSION PROFILING

A source of frustration surrounding our early work in this area was the need to run individual immunoassays on each protein of interest. This limited the number of analyses that could be run on each sample. More recently, multiplex immunoassays (e.g., Luminex beads) or assay services (e.g., Rules-Based Medicine, Austin, TX) have opened the door to more extensive analysis of adsorbed proteins or other biomarkers. One such application, looking at structural skin proteins and serological markers, was recently published by Hendrix et al. (26). They used D-Squame tapes to adsorb and quantify structural proteins (involucrin, fibronectin, keratins-1, -6, and -10) and plasma biomarkers (cortisol, human serum albumin) from healthy forearm skin. They used a multi-analyte-profiling method, SkinMAP^{TM} (Linco Research, St. Charles, MO). This initial study defined study design and extraction procedures for future work. It is likely that cytokine analysis will also be forthcoming via this method.

A recent study from the Netherlands used a more aggressive tape-stripping method with several different types of tape (including D-Squame) and examined baseline and SLS-induced cytokine recovery at three different levels from the outer stratum corneum to the lower stratum corneum just above the viable epidermis (27). Similar to our findings, they reported increased IL-1ra (and IL-1ra/IL-1α ratios) in chronically irritated (SLS-exposed) skin. This increase was consistent across all levels of the stratum corneum. A slight increase in IL-8 was also observed.

Another application of tape stripping of skin has been to recover mRNA fragments that code for inflammatory or other proteins. Morhenn et al. first reported on this technique in 1999, showing differential recoveries of RNAs coding for the cytokines IL-4 and IL-8 and the enzyme-inducible nitric oxide synthase (28). They recovered the RNA from multiple (up to 23) D-Squame tape strips of the skin and used a ribonuclease protection assay for detection and quantification. They showed distinctly elevated recoveries of RNA for all three proteins from allergic contact dermatitis skin sites versus irritant contact dermatitis skin sites. Later, using a more limited four-repeated tape-stripping procedure, they recovered and amplified mRNA from normal and SLS-irritated skin and examined gene expression profiles via microarray analysis (29). They demonstrated significantly altered expression in over 1700 genes as the result of SLS-induced skin irritation. In similar fashion, Benson et al. (30) used four tape strips from psoriatic skin sites or non-lesion sites and measured cytokine and keratin protein recoveries via RT-PCR analysis. Using this approach, they detected changes (overexpression) in biomarkers that were distinct from those seen via biopsy procedures and felt that the more noninvasive approach was a useful adjunct for the study of this disease.

CONCLUSION

The ability to study the inflammatory response of the skin has traditionally relied on invasive techniques to collect cells and mediators of this complex response. Skin surface adsorption of biomarkers of inflammation certainly does not tell the entire story of the underlying inflammatory processes. By the simple fact that adsorption is from the surface of the nonviable stratum corneum, the molecules collected were derived from earlier synthetic processes in response to some stimulus. This may make it difficult to easily discern (from the absorbed molecule profiles) the exact nature or mechanisms of acute inflammatory responses. However, chronic conditions or diseases, for which the molecular responses are ongoing, are much more assessable by this approach. The consistent finding (from multiple skin conditions, diseases, and across several laboratories over the years) of elevated IL-1ra/IL-1α ratios is a testament to the validity and utility of the approach and the value of noninvasive tape adsorption of biomarkers as a means to detect, differentially diagnose, and evaluate inflammatory skin conditions.

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46 Detecting Skin Irritation Using Enhanced Visual Scoring: A Sensitive New Clinical Method[a]

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INTRODUCTION

Skin testing on panels of volunteer human subjects to confirm that a new material is nonirritating is a routine part of the overall product safety assessment. Tests to assess irritation range from standard occluded patch tests to tests that exaggerate the concentrations of actual expected exposures. Currently, these tests rely mainly on unaided visual scoring of erythema to determine the degree of irritation.

As with any other type of inflammatory reaction, skin irritation triggers a series of events involving subsurface dilatation of blood vessels with an influx of inflammatory cells. Subsequently, erythema (along with possible swelling and heat) appears on the skin surface. However, by the time consumer products being developed reach the stage when they can be safely and ethically tested on human volunteers, products and ingredients that may cause frank irritation have been eliminated. The resulting products are virtually nonirritating and produce, at most, very minor visual changes, even under conditions of highly exaggerated exposures. This presents a difficult challenge when trying to differentiate between the potential skin effects of two closely related products. [For a more complete discussion on irritation, see Chew and Maibach (1).]

Recently, we evaluated the effectiveness of a polarized light visualization system that uses both parallel- and cross-polarized light. Our goal was to determine if enhanced visual scoring using a polarized light visualization tool would enable us to detect subclinical irritation, i.e., changes that occur before irritation is obvious to the unaided eye, or changes that may still be present after visible changes have resolved. If successful, this would lead to (*i*) increased sensitivity of our testing program enabling us to differentiate between very mild test products, (*ii*) improved claims support, and (*iii*) better guidance in our product development efforts. It would also enable us to design tests that use fewer exposures of shorter duration, resulting in programs that are less costly and cause less discomfort to our panels of volunteer subjects.

PROCEDURE

Two basic test designs were used: the standard patch test and the behind-the-knee (BTK) test. Typically, these tests would be conducted as a part of routine evaluation of the potential irritant properties of a material. The patch test measures the potential for irritation due to the chemical structure of a material (i.e., chemical irritation). The BTK test measures both chemical irritation and the potential for mechanical irritation due to friction (2–4). Scoring was conducted to compare unaided visual scoring to enhance visual scoring with a polarized light visualization system.

The test materials consisted of low concentrations of Sodium Lauryl Sulfate (SLS) and two currently marketed brands of feminine hygiene pads. Pad A is a standard pad with an absorbent core and a fabric-like, polyethylene film top sheet. Pad B is a standard pad with an absorbent core and a nonwoven fabric top sheet. These products are similar in their potential to cause irritation, yet consumers prefer product B over product A due to the absence of adverse skin sensations.

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[a]Same parts Adapted from Farage MA. Enhancement of visual scoring of skin irritant reactions using cross-polarized and parallel polarized light. Contact Dermatitis 2007; 57: 1–9. (With kind permission from Blackwell Publishing Group.)

For each study, the protocol was approved by the test facility's institutional review board. Participants in all the studies were healthy adult volunteers aged 18 to 65 years who had signed an informed consent. Subjects could withdraw from the study at any time. The studies were conducted in compliance with the Good Clinical Practices regulations as prescribed by the Food and Drug Administration (5).

All subjects had very sensitive to moderately sun-sensitive skin (types I–IV) according to Fitzpatrick's classifications (6). Subjects were excluded from participation if they (*i*) had sunburn, acne, scar tissue, dermatitis, or any other skin abnormality at the test sites, (*ii*) were taking anti-inflammatory corticosteroids or other medications that may interfere with test results, (*iii*) had participated in an arm patch test within the last four weeks, (*iv*) had diabetes or any medical condition that might compromise the immune system, or (*v*) were pregnant. Subjects were instructed to refrain from using lotions, creams, or any other skin preparation in the test area; to refrain from swimming and tanning/sun exposure; and to refrain from taking any anti-inflammatory, anti-histamine, and/or steroid medications while participating in the study.

Test materials were applied as follows.

Testing on the Upper Arms and Lower Forearms

Standard patch testing has been described previously (3). Samples were applied using an occlusive, nonwoven cotton pad (Webril patch, Professional Medical Products Company, Kendall LTP, Chicopee, Massachusetts). The adhesive was reinforced with an occlusive, hypoallergenic tape [Blenderm occlusive tape (3M Health Care, St. Paul, Minnesota)]. On the upper arm, patches were applied lengthwise midway between the shoulder and the elbow on the lateral surface, with a minimum of 2-cm space between patches. On the lower forearm, patches were applied lengthwise to the volar surface midway between the elbow and the wrist.

In the first experiment, samples were applied to the upper arm for 24 hr/day for three consecutive days. In the second experiment, patches were applied to both the upper arm and the lower forearm for either 2 or 6 hr/day for two consecutive days.

Patch sites for the different test materials were rotated among subjects. Separate patches were applied for sites that would be scored visually and sites that would be scored using the visualization instrument to avoid bias that might be introduced inadvertently by repeated grading of a specific site. Patch sites were marked with 0.5% gentian violet to aid in visual grading and to ensure that the patches were applied to identical sites each day for the duration of the test. Panelists were instructed to remove the patches at specific times and to return to the laboratory for grading and/or reapplication of test materials 30 to 60 minutes later. Further details of each experiment are included in the appropriate figure legend.

BTK Testing

The protocol was a modification of the BTK test described previously (2–4). The test material was placed horizontally and held in place behind the knee by an elastic knee band of appropriate size. Test materials were removed by the panelists 30 to 60 minutes prior to returning to the laboratory for grading and/or reapplication of materials. Samples were left in place for six hours each day, for four consecutive days.

Grading was conducted prior to the first patch application (i.e., baseline), and 30 to 60 minutes after removal of each patch (upper arm or lower forearm) or BTK application (i.e., "post patch"). In the BTK and short duration patches (2 or 6 hours on the upper arm or lower forearm), scoring was also conducted the following morning after sample removal, prior to the next sample application (i.e., "recovery").

Standard visual grading was done by a trained, expert grader under a 60 to 100 watt incandescent daylight blue bulb. Enhanced visual grading was conducted using a polarized light visualization system (Syris v600® Visualization System, Syris Scientific, LLC, Gray, Maine; available at: www.syrisscientific.com) with separate scores recorded for parallel-polarizing illumination (surface mode) followed by cross-polarizing illumination (subsurface mode). The subsurface mode allows visualization of the site at a depth of 1 mm beneath the surface.

Erythema was graded according to a previously described scale of "0" to "4", where 0 is no apparent cutaneous involvement and 4 is moderate-to-severe, spreading erythema and/or edema (2). The same grader was used throughout an experiment, and the grader was not

aware of the treatment assignments. If a test site exhibited a grade of "2" or greater, the test material was not reapplied at that site. However, the site was graded until completion of the test. Any test site showing a grade of 2 or more at the final grading timepoint was followed until the response regressed to a "1.5" or less.

For the BTK test, panelists kept a daily diary of skin problems experienced at the test sites, as described previously (7,8). Panelists were asked if they experienced any of the following sensations: the sample rubbing against the skin, the sample sticking to the skin, chafing, burning, itching, pain, edema, or any other discomfort. Results were evaluated for a higher occurrence of each individual skin problem with one treatment versus another.

Stratified CMH (Cochran-Mentel-Haenszel) comparisons were used to evaluate erythema scores, unless otherwise indicated. For sensory effects, treatment comparisons were evaluated using McNemar's test. Results of the statistical analyses conducted for each experiment are described in the appropriate figure legend.

SKIN IRRITATION ASSESSMENT

In the standard 24h-patch test: grades using polarized and cross-polarized light were similar to the grades resulting from standard visual assessment. The mean erythema grade after the first 24-hour patch application to the upper arm was statistically significantly different from the baseline grade for all three grading methods (visual, subsurface using cross-polarized light, and surface using parallel-polarized light) (Fig. 1). For all test concentrations of SLS (0%, 0.025%, 0.05%, and 0.1%), the mean erythema remained significantly elevated over baseline grade with subsequent patch applications. A repeat experiment using 24-hour patches of SLS at concentrations of 0.01% and 0.1% on the upper arm and 0.01% on the volar surface of the forearm showed similar results (data not shown).

Enhanced visual grading was more effective in detecting very minor irritation. Patch tests were conducted on the upper and lower arm under conditions that produce very low levels of irritation (e.g., concentrations of SLS of 0.01% and patch test exposures of 2 and 6 hours). Grading with cross-polarized light (subsurface grading) detected statistically significant increases in mean erythema compared with baseline after a single patch application for two hours or six hours on the forearm (Fig. 2A), and for six hours on the upper arm (Fig. 2B). With a patch exposure of two hours on the upper arm, the increase in subsurface erythema was not significant immediately after patch removal, but was significant after 22 hours of recovery (i.e., recovery 1).

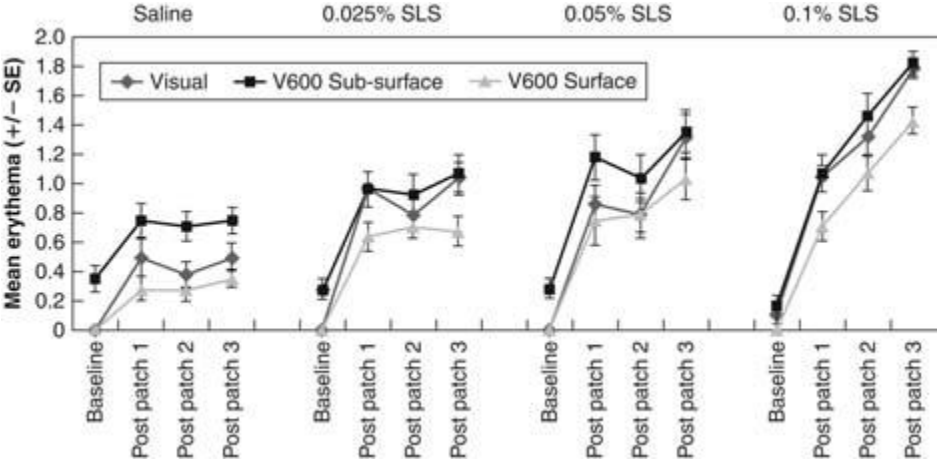

Figure 1 Standard visual and enhanced visual grading (24-hour patch test on the upper arm). Test samples in the 24-hour patch test consisted of saline, 0.025%, 0.05%, and 0.1% SLS. Panelists (13–14 per group) were patched on the upper arm for 24 hr/day for three consecutive days. Scoring was conducted at baseline (prior to treatment), and 30 to 60 minutes after removal of each patch (post patch 1–3). The graph plots mean erythema (±S.E.) at each scoring timepoint. Stratified CMH comparisons were used to evaluate visual scores for 0.05% and 0.1% SLS. ANCOVA was used for all other treatments. For all treatments, visual, subsurface and surface scores after patches 1 to 3 were significantly elevated over baseline ($p \leq 0.05$, not shown on graph). *Abbreviation*: CMH, Cochran-Mentel-Haenszel; ANCOVA, analysis of Co-variance.

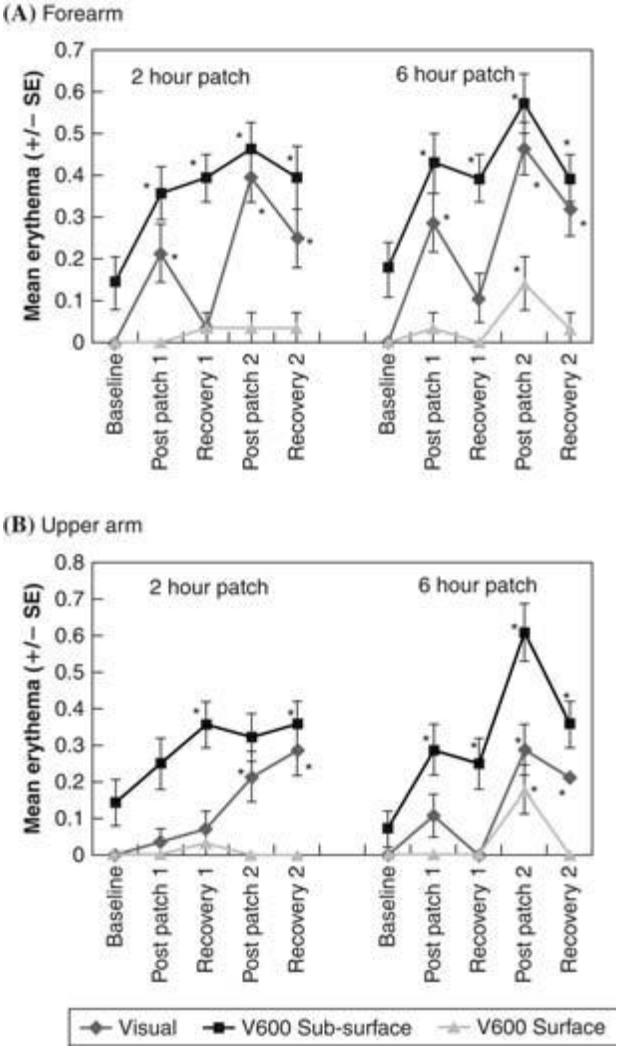

Figure 2 Standard visual and enhanced visual grading using very mild conditions in the patch test (lower forearm and upper arm). Multiple patches of the 0.01% SLS were applied to both (**A**) the forearm and (**B**) upper arm for two consecutive days (14 panelists per group). Patches were removed after two hours or six hours. Scoring was conducted at baseline, 30 to 60 minutes after removal of each patch (post patch 1–2), and the morning following each patch removal (recovery 1–2). The graph plots mean erythema (±S.E.) at each scoring timepoint. Results were evaluated using the stratified CMH test. (*Significant difference from baseline, $p \leq 0.05$). *Abbreviation*: CMH, Cochran-Mentel-Haenszel.

Similar results were obtained using 0.03% SLS, with patches applied for two and six hours (data not shown). Irritation was apparent earlier with the subsurface scoring (upper arm) and tended to remain elevated, even after overnight recovery.

Enhanced visual grading increased the ability to differentiate between two similar products in the BTK test. At the afternoon scoring, conducted immediately after removal of the first test sample (i.e., post–patch 1 scoring), the subsurface scores for the two products were significantly different and remained different for the subsequent post-patch scoring times. This difference was apparent using unaided visual scoring and surface scoring (Fig. 3A and C), and the subsurface assessment using cross-polarized light (Fig. 3B). However, at the scoring after the test sites recovered overnight (i.e., recovery scoring), significant differences between the two products were not apparent until after the third application for the visual scoring (recovery 3, Fig. 3D) and after second application for surface scoring (recovery 2, Fig. 3F). Subsurface scoring showed the products to be significantly different after one single application (Fig. 3E).

Enhanced visual grading results were consistent with sensory effects in the BTK test. Test panelists' diaries enabled evaluation of sensory effects. With every sample application, significantly more individuals reported burning sensations with pad A compared with pad B (Fig. 4). In addition, a significantly higher number of individuals reported pain with pad A during the third sample application and the sensation of the sample sticking to the skin during the second and third applications (data not shown).

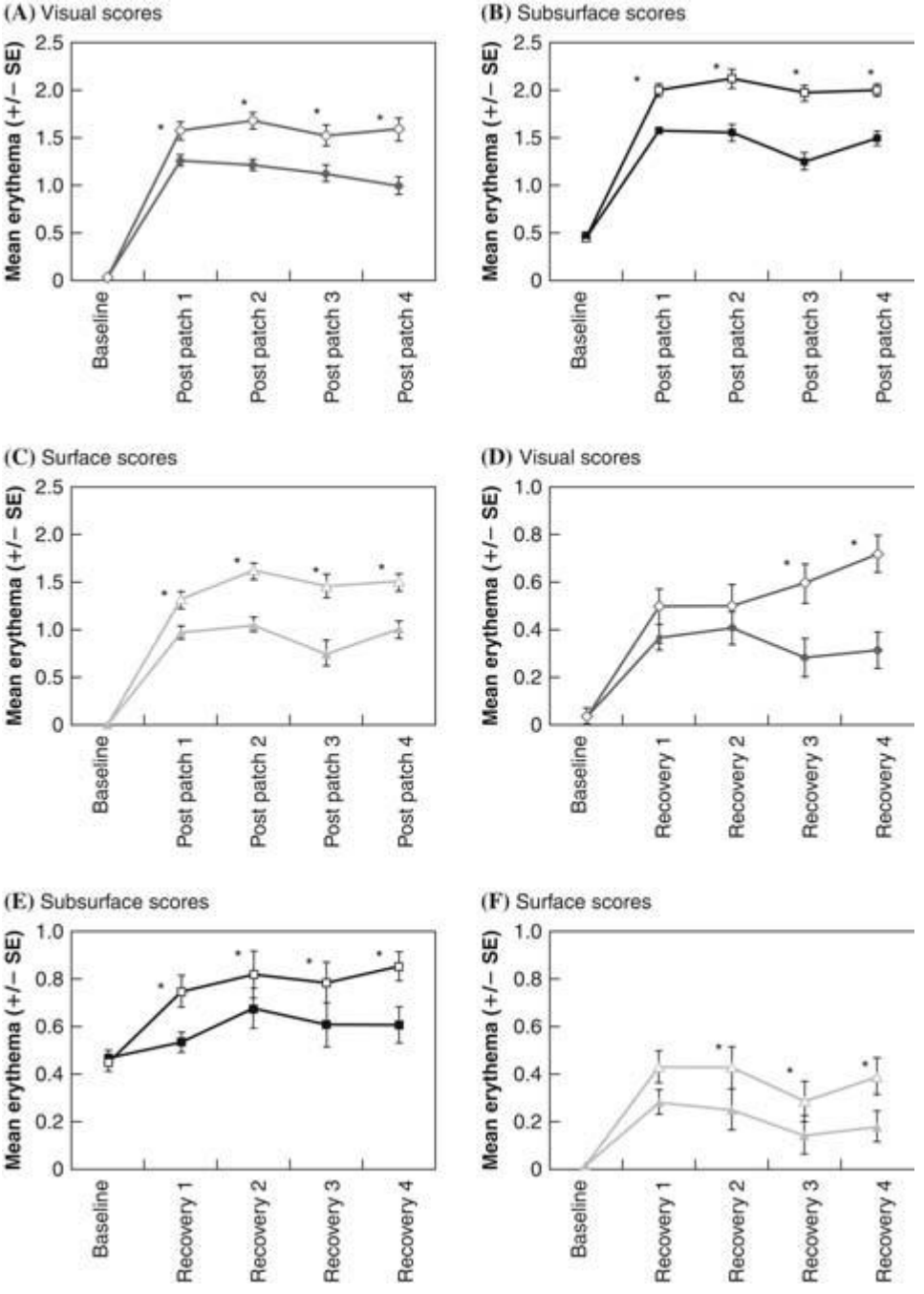

Figure 3 Standard visual and enhanced visual grading using two similar products in the BTK. Two feminine protection products (pad A and pad B) were evaluated in the BTK. Samples were applied for 6 hr/day for four consecutive days (14–16 panelists per group). Scoring was conducted at baseline, 30 to 60 minutes after removal of each patch (post patch 1-4, **A**–**C**), and the morning following each patch removal (recovery 1–4, **D**–**F**). The graph plots mean erythema (±S.E.) at each scoring timepoint. Treatment comparisons were evaluated using the stratified CMH test. (*Significant difference between pad A and pad B, $p \leq 0.05$). Pad A = open symbols. Pad B = closed symbols. *Abbreviations*: BTK, behind-the-knee; CMH, Cochran-Mentel-Haenszel.

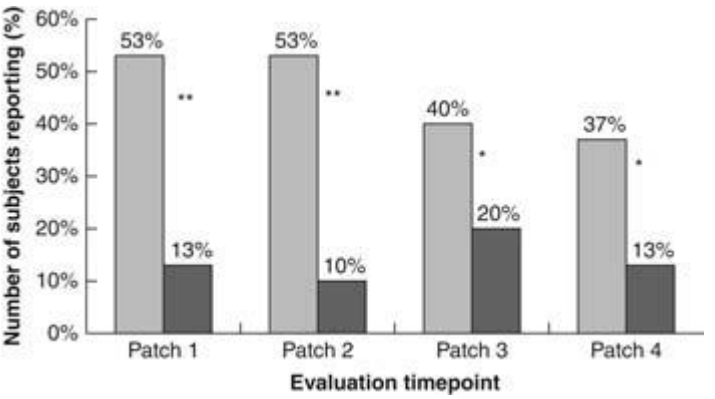

Figure 4 Reports of burning sensations in the BTK. In the BTK, each of the 30 panelists was asked to keep a daily diary of skin problems experienced at the test sites. Results were evaluated for a higher occurrence of each individual skin problem with one treatment versus another. The graph plots the number of subjects reporting sensations of burning at the test sites during each patch application. Treatment comparisons were evaluated using McNemar's test. (**Significant difference between pad A and pad B, $p < 0.001$). (*Significant difference between pad A and pad B, $p < 0.05$). Pad A = open symbols. Pad B = closed symbols. *Abbreviation*: BTK, behind-the-knee.

DISCUSSION

Evaluating the potential for skin irritation is an important step in assuring the safety of many consumer products. Visual grading of erythema has been used reliably for many years to detect skin irritation in a variety of test protocols and on various body sites. It requires no special equipment and is easily adaptable to large-scale testing, which is required to provide safety assurance for consumer products. Trained skin graders can accurately and reproducibly score test sites for erythema and dryness (9) and reliably detect evidence of irritation with equal or higher degrees of sensitivity to that of instrumental measures (10–14). However, enhanced visual scoring may increase the ability to differentiate very similar products without requiring other protocol modifications. In addition, by continuing to use erythema as a signal for skin irritation, we can compare data on new materials and products with a large, historical data set.

Current test methods were developed so a grader would see a reaction with the unaided eye. However, physiological changes that occur early in the process of irritation, such as changes in blood flow, moisture content, and pH, would be expected to occur before any reaction is visible. In other words, by the time the reaction is visible, it may be too late to measure the early changes in skin physiology. These early changes may be key to our ability to distinguish subtle skin effects and, therefore, support future product development efforts.

When skin reactions are scored visually, the grader is seeing a combination of endpoints: the surface changes, which provide information about the shape and texture of the skin surface, and the subsurface changes, which provide information about internal components such as erythema, pigmentation, and the vasculature (15,16). Polarized light sources can enable the observer to selectively examine either the surface or subsurface components.

Authors have described the use of polarized light as an aid in visualizing various skin conditions, including acne vulgaris, rosacea, photoaging, lentigo simplex, and basal cell carcinoma (17–19). Kollias et al. (20) studied irritation reactions in individuals' patch tested with various concentrations of SLS. After 24 hours of patch testing, test sites were photographed with standard and perpendicular polarized (or cross polarized) light 20 minutes, 24, and 48 hours after patch removal. Visible erythema was evaluated on both sets of photographs. These authors found that erythema was apparent at the 20-minute photograph and persisted for the next 48 hours.

We conducted these studies to determine if the use of cross-polarized light would increase the sensitivity of our scoring and allow us to detect reactions that were not apparent with unaided visual grading. In the first experiment, the standard, 24-hour patch test to the upper arm was used (Fig. 1). The low concentrations of SLS produced very low levels of irritation, as indicated by the relatively low mean erythema scores. All concentrations,

including saline alone, were sufficient to produce enough erythema so that the results were significantly different from the pre-patch (baseline) scores after the first 24-hour patch.

Evaluation of irritation reactions by expert graders has been used very effectively for decades in protocols designed to investigate skin effects (10,13,21–25). In an earlier publication, we conducted direct comparisons between visual scoring and instrumental scoring methods [TEWL using a ServoMed Evaporimeter EP1®(Servomed AB, Stockholm, Sweden), and redness using a Minolta Chromameter CR-200®(Minolta Corp., U.S.)] (26). The results of the visual scoring method were very similar to those of the instrumental scoring methods. Significant differences between treatments and scoring timepoints of visual scores were very consistent with those observed with the instrumental scores. The results presented in Figure 1 provide a comparison with an instrument designed to enhance visual scoring (Syris v600® Visualization System). Once again, results are very consistent and confirm that an expert grader can detect very low levels of irritation. Results of the experiment presented in Figure 1 indicated that, although the test conditions were mild, the 24-hour patch on the upper arm produced irritation reactions that were already visually apparent, i.e., the reactions were not subclinical. Therefore, a subsequent experiment was designed using even milder conditions in an attempt to produce subclinical changes.

Results of the milder patch conditions (Fig. 2) demonstrated that, using enhanced visual scoring, irritant effects were apparent at certain timepoints in the study when standard visual scoring showed no significant effect. A low concentration of SLS (0.01%) was patched for very brief periods (2 hours and 6 hours). On the upper arm, the test conditions did not produce significant visible erythema until after the second patch. However, grading the subsurface reactions using cross-polarized light demonstrated significant erythema after removal of the first 6-hour patch, or 24 hours earlier (Fig. 2B).

When 0.01% SLS was patched on the volar forearms, the mean erythema was significantly different from baseline after a single patch using both standard visual scoring and cross-polarized light (subsurface) scoring. However, using standard visual scoring, the erythema disappeared with an overnight recovery (Fig. 2A, recovery 1). Yet, using cross-polarized light showed that subsurface changes in erythema were still present under the skin surface. The ability of the enhanced visual scoring to detect subclinical irritation may indicate that the use of this tool has the potential to increase the sensitivity of standard tests.

The results shown in Figure 3 demonstrate the challenge in detecting differences in the potential for irritant effects between two very similar products and the potential usefulness of the enhanced visual scoring. Pads A and B produce indistinguishable irritant reactions when tested in the standard 24-hour patch test on the upper arm. However, consumers and panelists who participate in skin effects' studies with these two products consistently report a higher number of unpleasant sensations, i.e., itching, sticking, etc., associated with pad A (7,8). The BTK test, which was developed to evaluate the potential for both chemical and mechanical irritation, has been the only test method to consistently detect a difference in the irritation potential of these two products (3,7).

In this study, we also demonstrated a significant difference between these two products after the first six-hour application in the BTK test (Fig. 3A–C). However, after an overnight recovery period, the difference between erythema using the standard visual scoring method was no longer significant (Fig. 3D). Using cross-polarized light to grade the subsurface reactions, the changes in erythema produced by pads A and B remained statistically significantly different, even after overnight recovery.

Enhanced visual scoring enables detection of subclinical physiological changes that are not apparent using standard visual scoring. We have reported previously that subjective consumer comments indicate that consumers can detect differences in skin effects caused by the use of two similar products. For example, consumers have consistently indicated that pad B is seen as less irritating than pad A in "real use" situations; however, most test protocols repeatedly fail to differentiate between these two products (3). The BTK test, which was developed to further exaggerate exposure due to the mechanical irritation component, is the only protocol that consistently differentiates between these two products.

Sensory effects have been shown to be consistent with BTK test results and to differentiate between pad A and pad B reliably (8). This current investigation confirms that sensory effects correlate with visual scoring in the BTK test and confirms that sensory effects enable the differentiation between two very similar products (Fig. 4). With enhanced visual

scoring, we can begin to bridge the gap in sensitivity between sensory effects and physiological changes that can be evaluated by an external observer.

The ability of enhanced visual scoring to detect irritant-related changes, even when standard visual scoring indicates recovery of the skin, has important implications for testing protocols. Currently, standard protocols on potential skin effects do not focus on recovery. It is assumed that a resolution of the erythema and other visual changes indicate an end to the irritant reaction. However, with enhanced visual scoring, subclinical changes are still evident, even with the mild reactions seen in this series of experiments. Future protocols can be designed to follow the subsurface changes to their resolution more effectively and to provide a clearer understanding of the process of skin healing.

CONCLUSIONS

We have developed a new innovative clinical method with several advantages:

- Subclinical skin irritation can be detected using enhanced visual scoring.
- Enhanced visual scoring indicates that subsurface changes to the skin persist, even when standard visual scoring indicates recovery.
- Enhanced visual scoring has the potential to increase the sensitivity of our clinical studies by detecting changes that are not apparent with standard visual scoring.
- Skin irritation is apparent with fewer applications of the test material. This may allow development of shorter protocols for evaluating the skin effects of consumer products.
- Enhanced visual scoring has the potential to bridge the gap in sensitivity between sensory effects experienced by the consumer and physiological changes that can be evaluated by an external observer.

This new method has the potential to increase the sensitivity of all clinical dermatological studies.

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47 Sodium Lauryl Sulfate–Induced Irritation in the Human Face: Regional and Age-Related Differences

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Although extensively studied (1), sodium lauryl sulfate (SLS) has been rarely used on the face to investigate mechanisms of irritation (2). Because of the particular skin sensitivity of the face and the neck and because of the regional variability in the face reactivity to water-soluble irritant compounds (3) and to compounds inducing contact urticaria (4,5), we conducted this study with SLS 2% under occlusion for one hour.

Because baseline transepidermal water loss (TEWL) has been speculated as a predictive parameter to skin susceptibility to SLS (6) and changes in hydration of superficial epidermis suspected to be responsible for the seasonal variability of skin irritation induced by SLS (7), we measured the baseline TEWL and capacitance before SLS application and studied their correlation with changes in TEWL (∂TEWL), 1 hour and 23 hours after patch removal (1).

INVESTIGATIONAL STUDY

Two age groups were examined: 10 young subjects, aged 25.2 $\pm$ 4.7 years ranging from 19 to 30 years and 10 older subjects aged 73.7 $\pm$ 3.9 years ranging from 70 to 81 years. Twelve volunteers were Caucasians and eight Hispanics.

Eight areas of the skin (forehead, nose, cheek, nasolabial and perioral areas, chin, neck, and volar forearm) were studied.

After 15 minutes of rest, necessary to suppress excess water evaporation, baseline TEWL was measured using an evaporimeter, Tewameter® TM 210* (Courage + Khazaka, Cologne, Germany) and baseline capacitance was measured with a Corneometer® CM 820 PC (Courage + Khazaka, Cologne, Germany).

SLS (Sigma, St. Louis, Missouri, U.S.) 2% (w/v) in water was then applied to each of the eight areas for one hour under occlusion, using a saturated absorbent filter paper disc (0.8 cm diameter) in small Finn Chamber aluminum discs (Epitest Ltd. Oy, Finland). On the contralateral side, water was applied in the same conditions as control.

To assess skin irritation, TEWL was measured 1 hour and 23 hours after patch removal.

TEWL values of the areas tested were corrected according to the changes in the control areas:

$$\text{TEWL} = \text{TEWL measured} - \text{TEWL } H_2O,$$

where TEWL measured is that in the tested area at 1hour or 23 hours, and ∂TEWL H_2O = TEWL control – baseline TEWL H_2O, where TEWL control is the measured TEWL value in the control area at 1 hour or 23 hours.

The skin reactivity to SLS was assessed by the changes in TEWL (∂TEWL = TEWL – baseline TEWL).

To compare the skin reactivity (∂TEWL) of the regions within each group, the two-tailed Student *t* test for paired data was used. The two-tailed Student *t* test for unpaired data was used to compare the two age groups.

Simple linear regression and correlation analysis between basal TEWL and skin irritation (∂TEWL) and between baseline capacitance and ∂TEWL for each skin location combining the data of the two age groups were used. ∂TEWL was considered as the dependent variable.

Table 1 Reactivity of Regions in the Young and Old Group

Area	∂TEWL (Mean ± SD) g/m^2 hr		*p* value
	Young group	Old group	
Cheek	15.1 ± 12.8	6.8 ± 7.3	0.093
Chin[a]	13.5 ± 9.9	6.0 ± 3.3	0.035
Forearm	1.9 ± 2.1	1.1 ± 1.5	0.354
Forehead	10.4 ± 13.9	2.3 ± 2.3	0.086
Neck	6.8 ± 6.0	3.6 ± 3.7	0.165
Nasolabial area[a]	12.4 ± 6.3	4.4 ± 4.8	0.005
Nose	8.6 ± 7.6	5.0 ± 6.0	0.251
Perioral area	10.7 ± 10.0	4.2 ± 4.1	0.074

Note: ∂TEWL = TEWL 23 hours after patch test removal corrected to the control – baseline TEWL
[a]Difference between the young and old group statistically significant ($p < 0.05$).

COMPARISON OF REACTIVITY BETWEEN AGE GROUPS AND FACE AREA

SLS 2% under occlusion for one hour induced in most of the cases a subclinical irritation and sometimes minimal erythema. The absolute TEWL values taken after 1 hour and 23 hours did not show significant differences. Since the 23-hour measurements demonstrated lower standard deviation values, only the irritation assessed at 23 hours was considered.

Comparison Between the Regions

In the young group, all areas except the forearm reacted to SLS. Skin irritation induced by SLS and assessed by ∂TEWL was greater in the cheek and chin when compared with that the neck and forearm ($p < 0.05$). The highest ∂TEWL mean values were found in the cheek and chin (Table 1), but no statistically significant differences with the remaining regions of the face were detected. All the other regions except the forehead showed a significantly higher irritation than the forearm.

In the old group, all regions reacted to SLS except the nose, perioral area, and forearm. The cheek and chin showed the highest ∂TEWL mean values (Table 1).

Significantly ($p < 0.05$) higher reactivity of these two areas was found when compared with that in the forearm, and when the reactivity in the chin was compared with that in the forehead.

Comparison Between the Two Age Groups

In all the areas studied, the mean ∂TEWL values were higher in the young than in the older group (Table 1). Only in the chin ($p = 0.035$) and nasolabial area ($p = 0.005$) were the differences significant.

CORRELATION STUDY BETWEEN MEASUREMENTS

Correlation Between Baseline TEWL and ∂TEWL

Table 2 summarizes the correlations in each area between baseline TEWL and ∂TEWL 23 hours after patch removal.

The forehead and the neck showed the strongest correlations ($r = 0.6474$, $p = 0.002$ in the forehead; $r = 0.6273$, $p = 0.003$ in the neck).

The nose and chin did not demonstrate a significant correlation between basal TEWL and TEWL changes induced by SLS. The forearm was not studied since this area did not react to the surfactant in the same test conditions.

Correlation Between Baseline Capacitance and ∂TEWL

The baseline capacitance was not correlated to the skin irritation induced by SLS in any area studied.

Table 2 Correlations in Each Area Between Baseline TEWL (BTEWL) and Reactivity of the Skin to SLS, 23 hours after Patch Removal (∂TEWL)

	BTEWL (mean ± SD)	TEWL 23 hr (mean ± SD)	∂TEWL (mean ± SD)	*r*	*p*
Cheek[a]	15.63 ± 6.70	26.63 ± 15.30	10.96 ± 11.01	0.4616	0.040
Chin[a]	20.87 ± 6.37	30.47 ± 12.08	9.77 ± 8.13	0.3535	0.126
Forearm	8.64 ± 3.97	9.70 ± 4.92	1.51 ± 1.83	–	–
Forehead[a]	14.10 ± 5.71	20.40 ± 14.96	6.39 ± 10.53	0.6474	0.002
Neck[a]	11.55 ± 4.35	16.63 ± 8.54	5.18 ± 5.12	0.6273	0.003
Nasolabial[a]	28.74 ± 8.56	36.93 ± 13.44	8.40 ± 6.78	0.4831	0.031
Nose[a]	19.04 ± 6.03	25.27 ± 11.15	6.77 ± 6.92	0.3218	0.166
Perioral	24.25 ± 8.93	29.98 ± 14.6	7.47 ± 8.17	0.4547	0.044

Note: *r* coefficient of correlation
p significance (significant correlation when $p < 0.05$)

[a]Areas that reacted to SLS: statistically significant ($p < 0.05$) difference baseline TEWL and TEWL 23 hours after patch removal.

CONCLUSIONS

SLS, an anionic surfactant is widely used to study the sensitivity of the skin to irritants. Little information on the susceptibility of the face to SLS is available (2).

In this study, the influence of age and regional variability on SLS irritation was investigated with a focus on the skin of the face.

Only TEWL 23 hours after patch removal was taken into consideration because of the lower standard deviation (SD) when compared with the one-hour values. This difference in SD might be explained by the "transient damage to the water barrier of the skin" described by Agner and Serup (8) and induced by exposure to water. This transient increase of TEWL not related to SLS or to the evaporation of additional water lasts between 1 and 3 hours after patch removal.

Considering the increase of TEWL after SLS exposure (∂TEWL), the young group had a higher irritant response than the old group in the chin and nasolabial area. In the remaining regions including the neck, ∂TEWL mean values were higher in the young group, although the differences were not significant. This lack of significant differences might be explained by the high SD values (Table 1) in these regions. Previous studies (2,9) investigated the influence of age on the susceptibility to SLS and reported a decrease in sensitivity in the elderly, which is in concordance with our results.

Various protocols (concentrations, application time) use SLS in water solution to induce skin irritation (10,11). In our study, since the face was suspected to be more sensitive than the remaining regions of the body, and for a practical purpose, SLS 2% was applied only for one hour under occlusion. This protocol was sufficient to induce subclinical irritation in most of the areas of the face but not in the forearm, confirming that the face is more sensitive than the forearm.

Although the cheek and chin showed the highest ∂TEWL mean values, no regional variations were detected between the various regions of the face in both age groups, but the cheek and chin were more sensitive than the neck in the young group. This lack of significant differences between regions might be explained by the high SD observed in ∂TEWL values.

To see whether significant differences in skin irritation induced by surfactant exist between the regions of the face, higher SLS concentrations as well as repeated open applications should be tested.

Skin sensitivity to water-soluble irritants has also been explored by the stinging test (3,12). Marked regional variation in the intensity of stinging was found (3): nasolabial fold > cheek > chin > retroauricular area > forehead. However, stinging test expresses the percutaneous penetration of the irritant compound and the sensory nerve response that depends upon nerve density in the skin. Marked regional variability of the nerve density has been reported (13).

Skin irritation expressed by ∂TEWL is the result of percutaneous penetration of the compound and the changes made to the skin barrier. This could explain that both methods (stinging test and SLS-induced irritation) might not show the same sensitivity regional

variation in the human face, although the cheek and the chin were among the most sensitive areas demonstrated by both methods.

The correlation study showed a significant correlation between basal TEWL and ∂TEWL in five of the seven areas that have reacted to SLS (Table 2).

The correlations between baseline TEWL and TEWL 23 hours after patch removal were more obvious. All the areas that reacted to SLS (all the areas studied except the forearm) showed a strong correlation coefficient varying between 0.76 and 0.88, with a highly significant p value <0.001.

However, we think that the correlation between basal TEWL and the absolute TEWL values after irritation does not imply that higher basal TEWL values predispose to higher skin sensitivity, but only the correlation between baseline TEWL and the changes in TEWL after irritation (∂TEWL) may have this significance. Even if for different basal TEWL values, the changes in TEWL are the same, a positive correlation could be found because TEWL is considered as a stable parameter (14).

Conflicting results have been published with regard to this aspect. Some authors correlated the absolute TEWL values before and after irritation (6,15–17). Others (18) used basal TEWL and ∂TEWL. Agner (18), studying healthy and atopic subjects, reported a positive correlation between baseline TEWL and the increase in TEWL induced by SLS only in the healthy group. Although in the atopic group, basal TEWL was significantly higher than the normal subjects, the changes after SLS exposure were not significantly different between the two groups.

These findings are in concordance with our study, where some areas of the face (nasolabial area) showed higher basal TEWL values than others (the cheek) but failed to demonstrate higher sensitivity (Table 2). So, each region of the face has probably its own characteristics influencing the skin sensitivity to irritants, probably independent, from basal TEWL.

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48 Irritation Differences Between Genital and Upper Arm Skin and the Effects of Emollient Application[a]

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The vulva comprises variable, specialized tissues that differ in structure, morphology, and embryonic derivation. The cutaneous epithelium of vulvar structures, including the labia major and labia minora, is keratinized. The epidermis of the labia majora contains sweat glands, sebaceous glands, and hair follicles; these structures are absent in the labia minora.

The labia majora has greater cutaneous thickness and keratinization than the labia minora.

The vulvar structures are highly vascularized, with the labia majora exhibiting more than twice the blood flow as in forearm skin (1).

Menses and venous blood differ in many factors of their composition. Approximately 50% of menstrual fluid is blood; the remainder of the menses fluid is made up of desquamated endometrial tissue and vaginal epithelial cells, cervicovaginal secretions, and endogenous vaginal microbes. The hemoglobin and iron content of menses varies throughout the menstrual cycle and is generally higher than in venous blood, whereas white blood cell and platelet counts are lower in menses than in venous blood (2).

The tissues of the labia majora and minora are more permeable than tissues at other anatomical sites, such as the forearm. To assess whether menses exposure contributes to vulvar irritation, we performed a four-day skin patch test of menses and venous blood on the labia majora and on the upper arm. To our knowledge, the potential contribution of menses to vulvar irritation has not been examined previously by skin patch test methods. In the study presented here, menses and venous blood have been compared to determine whether components unique to menses fluid (e.g., the matrix metalloproteinases, enzymes that catalyze endometrial breakdown) (3,4), might contribute to skin irritation. Although patch testing is performed routinely on the back or the arm, we chose to assess both the arm and the vulva because anatomical differences in irritant susceptibility can affect the erythema response (5,6).

The study that has been conducted was approved by an Institutional Review Board. All 20 women volunteers provided written informed consent. Physiologic saline (nonirritant control), aqueous sodium lauryl sulfate (SLS, 0.6% w/v, irritant control), and each volunteer's own venous blood and menses collected overnight with an intravaginal cup (Instead Softcup®, Ultrafem Inc., Missoula, Montana, U.S.) (0.3 mL each) were applied for two consecutive 24-hour periods to the lateral labia majora (randomized across 2 clipped sites on each labium) and to the upper arm (randomized across 5 sites per arm, see below). Occlusive patches (Webril® cotton pad, Professional Medical Products Company, Kendall LTP, Chicopee, Massachusetts, U.S.) secured with Blenderm® occlusive tape (3 M Health Care, St. Paul, Minnesota, U.S.) were applied to the labia and to one upper arm; semi-occlusive patches (Finn Chamber®, Epitest, Hyria, Finland) secured with Tegaderm® tape (3 M Health Care, St. Paul, Minnesota, U.S.) were applied to the alternate arm. The fifth site on each arm was pretreated with a proprietary, petrolatum-based emollient prior to menses application. A standard five-point erythema scale was used to score skin irritation (7,8).

This study showed that the labia majora were less responsive than the upper arm to all applied materials (Fig. 1A and B). Notably, menses and venous blood elicited no significant

[a]Some parts adapted from Farage M, Warren R, Wang-Weigand S. The vulva is relatively insensitive to menses-induced irritation. Cutan Ocul Toxicol 2005; 24(4):243–246. With kind permission from Taylor and Francis Group.

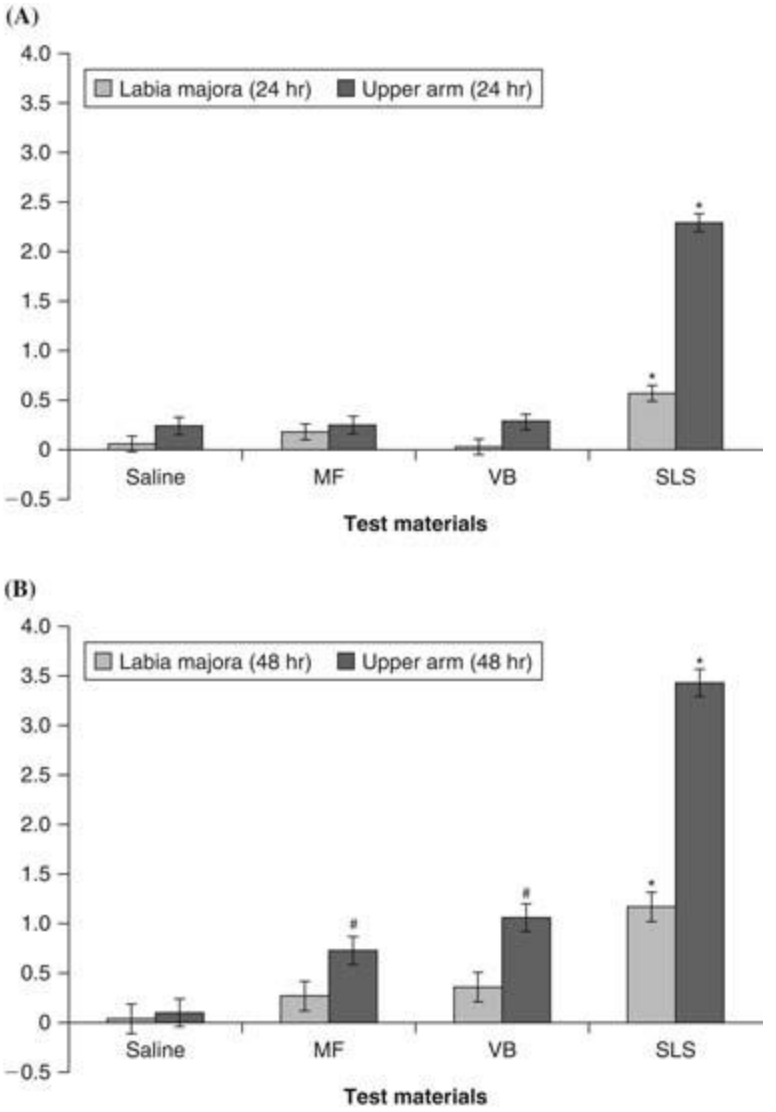

Figure 1 Skin erythema of the labia majora and upper arm following test materials application under occlusive patch for 24 and 48 hours, respectively. *Abbreviations*: Test materials: Saline, nonirritant control; MF, menses fluid; VB, venous blood; SLS, 0.6% aqueous sodium lauryl sulfate (irritant control). (**A**) 24-hour exposure. *: Significantly different ($p \leq 0.05$) from other test materials applied to that anatomical site. (**B**) 48-hour exposure. #: Significantly different ($p \leq 0.05$) from the nonirritant control (saline) applied to that anatomical site. *: Significantly different ($p \leq 0.05$) from other test materials applied to that anatomical site.

erythema on the labia majora at either time point; SLS, the irritant control, elicited significant, mild erythema (0.6 ± 0.08 and 1.2 ± 0.15 at 24 and 48 hours, respectively). The limited response to menses and venous blood on the labia could not have been predicted a priori. Indeed, some polar substances (e.g., benzalkonium chloride, maleic anhydride) elicit heightened irritant reactions on the labia relative to the arm (5).

On the upper arm, menses and venous blood elicited mild erythema at the 48-hour time point only (Fig. 1B: 0.7 ± 0.14 and 1.1 ± 0.14, respectively). No discernible difference in skin irritation to these fluids was observed at this site, despite differences in composition between menses and venous blood. Sodium lauryl sulfate elicited moderate to severe erythema on the arm at both the 24- and 48-hour time points (Fig. 1A and B: 2.3 ± 0.09 and 3.4 ± 0.14, respectively). Mean scores to SLS application on the arm were three- to fourfold higher than

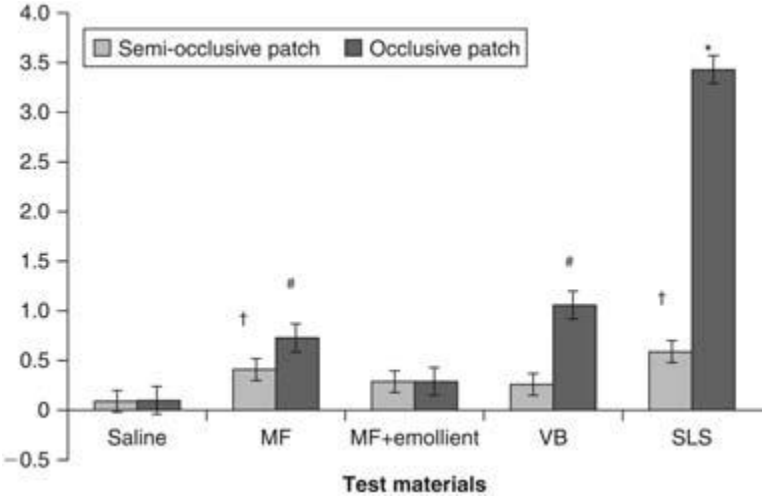

Figure 2 Skin erythema of the upper arm induced by test materials applied for 48 cumulative hours under semi-occlusive or occlusive patch. *Abbreviations*: Test materials: Saline, nonirritant control; MF, menses fluid; MF + emollient, menses fluid applied to emollient treated skin; VB, venous blood; SLS, 0.6% aqueous sodium lauryl sulfate (irritant control). †: Significantly different ($p \leq 0.05$) from the nonirritant control (saline) under semi-occlusive conditions. #: Significantly different ($p \leq 0.05$) from the nonirritant control (saline) under occlusive conditions. *: Significantly different ($p \leq 0.05$) from other test materials under occlusive conditions.

those observed on the labia; this is consistent with prior reports that the arm is more susceptible to SLS-induced skin irritation than the labia (6,9).

Semi-occlusive conditions attenuated the erythematous response to all materials (Fig. 2, upper arm, 48 hours). Notably, SLS-induced erythema was reduced almost sixfold (mean scores of 0.6 ± 0.1 vs. 3.4 ± 0.14, semi- and full-occlusion, respectively). Pretreatment of the upper arm with emollient prevented menses-induced skin irritation, regardless of the degree of occlusion (10).

CONCLUSIONS

To our knowledge, the potential contribution of menses to vulvar irritation has not been examined previously by skin patch test methods. Our preliminary observations suggest that the vulva (labia majora) is adapted to be less sensitive to menses-induced skin irritation. This adaptation is not universal, as other irritants have elicited heightened responses on the vulva (5).

We also found that pretreatment with a petrolatum-based emollient attenuates potential skin irritation from menses. If the latter observation can be extended to other biological fluids, it may be clinically relevant to limiting skin irritation under wound dressings, sanitary pads, and incontinence garments.

One caveat to interpreting visually scored skin erythema at these anatomical sites: heightened vulvar pigmentation as compared with the arm may mask inflammation, and utilizing bioengineering technology may clarify this issue. Future studies should also examine whether the menstrual cycle has an impact on the irritant response. Skin barrier function and reactivity to irritants at other sites exhibit cyclical variability (11,12), but an effect of the menstrual cycle on vulvar skin reactions has not been documented.

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49 Ethnicity as a Possible Endogenous Factor in Irritant Contact Dermatitis: Comparing the Irritant Response Among Caucasians, Blacks, and Asians

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INTRODUCTION

Irritant contact dermatitis (ICD) is a common and potentially serious dermatological disorder (1–3). It is also the second most common occupational illness (4). Since contact dermatitis can develop into chronic skin disease, understanding the underlying factors of its etiology is clinically important.

This condition is divided into several forms, depending on the nature of exposure and the resulting clinical presentation. Two common entities are acute and cumulative dermatitis. Acute contact dermatitis presents the classic symptoms of irritation such as localized and superficial erythema, edema, and chemosis. It occurs as a result of single exposure to an acute irritant (5). Cumulative irritant dermatitis presents similar symptoms but occurs when exposure to a less potent irritant is repeated until signs and symptoms develop over weeks, years, or decades.

The ability of the offending irritant to cause dermatitis depends on both the nature of the irritant agent and the initial skin condition. The severity of symptoms depends on exogenous and endogenous factors (6–8). Exogenous factors include the irritant's chemical and physical properties and the vehicle and frequency of application. Endogenous factors have been speculated to be age, sex, preexisting skin diseases, skin sensitivity, genetic background, and—the subject of this review—race (6), or, in today's parlance, ethnicity.

Ethnic differences in skin physiology and pathophysiology exist (9–11), and so whether ethnicity, is in fact, an endogenous factor affecting ICD is an important question in dermatotoxicology. Ethnic predisposition to ICD has been studied by comparing the irritant responses of blacks and Asians to those of Caucasians as a benchmark. We review these studies to evaluate if ethnic differences in susceptibility to ICD do exist.

The answer to the question of ethnicity as a factor in ICD has clinical and practical research consequences. Premarket testing of topical products (soaps, detergents, perfumes, and cosmetics), risk assessment for occupational hazards, and subject-inclusion requirements for product safety studies require knowledge about ethnic differences in irritation (12).

BLACK VS. CAUCASIAN IRRITATION RESPONSE

Using erythema as the parameter to quantify irritation, early studies note that blacks display less redness than Caucasians. In a hallmark paper, Marshall et al. (13) showed that while 59% of Caucasians exhibit acute irritant dermatitis as defined by erythema from 1% dichlorethylsulphide (DCES), only 15% of blacks do. Later, Weigand and Mershon (14) performed a 24-hour patch test using orthochlorobenzylidene malononitrile as an irritant, which confirmed that blacks are less susceptible than Caucasians to ICD as defined by erythema (Table 1, item A). Further studies, also using erythema as a measure of irritation, showed that blacks are less reactive than Caucasians to irritants (160 and 1280 mM/L methacoline) (19,20).

Weigand and Gaylor (21) showed that if the stratum corneum (SC) of black and Caucasian subjects is removed, there is no significant difference in irritation as measured by erythema between the two groups. They concluded that there might be structural differences

Table 1 Black Vs. Caucasian Irritation Response

Interference	Endpoint	Comment	Reference
A. Findings that show a statistically significant difference in the irritation response between blacks and Caucasians:			
1% Dichlorethylsulfide	Erythema	Untreated	Marshall et al. (13)
Orthochorobenzylidene	Erythema	Untreated	Weigand et al. (14)
100-mM methyl nicotinate	PPG	Untreated	Guy et al. (15)
0.05% Clobetasol	LDV	Pre-occluded	Berardesca and Maibach (16)
0.5–2.0% SLS	TEWL	Pre-occluded	Berardesca and Maibach (17)
B. Findings that do not show a statistically significant difference in the irritation response between blacks and Caucasians:			
0.5–2.0% SLS	LDV and WC	Untreated, pre-occluded, and pre-delipidized	Berardesca and Maibach (17)
100-mM methyl nicotinate	LDV	Untreated	Guy et al. (15)
0.1-, 0.3-, and 1.0-M methyl nicotninate	LDV and Erythema	Untreated	Gean et al. (18)

Abbreviations: PPG, photoplethysmography; LDV, laser Doppler velocimetry; TEWL, transepidermal water loss; WC, water content.

in the SC that provide more protection from chemical irritation to black skin than Caucasian skin. Indeed, while the SC thickness is the same in both races (22), the SC of black skin has more cellular layers and stronger cells (12), more casual lipids (23), increased desquamation (24), decreased ceramides (25), and higher electrical resistance (26) than Caucasian skin. Wesley and Maibach also found significant evidence that innate differences exist in skin properties between black and Caucasian skin (3). They found that blacks had higher TEWL (transepidermal water loss) values, decreased skin surface pH, variable blood vessel reactivity, and large mast cell granules. These variables, they concluded, may play a role in the differences observed in dermatologic skin disorders between blacks and Caucasians.

It is difficult, however, to conclude that blacks are less susceptible to cutaneous irritation only on the basis of studies using visual scoring. Erythema is notoriously difficult to measure in darker skin. Perhaps the difference in skin irritation between the two test groups is simply a result of the difficulty of assessing erythema in black subjects.

To better understand this issue, it is necessary to analyze studies that use alternative accurate detection methods (27) to assess the level of induced cutaneous irritation. Berardesca and Maibach (17) performed such a study to determine the difference in irritation between young Caucasian and young black skin. They applied 0.5% and 2.0% sodium lauryl sulfate (SLS) to untreated, pre-occluded, and pre-delipidized skin. Then they quantified the resulting level of irritation using objective techniques: laser Doppler velocimetry (LDV), TEWL, and water content (WC) of the SC. They found no statistical difference in irritation between the two groups as measured by LDV and WC, but they did find a statistical difference in the TEWL results of the pre-occluded test with 0.5% SLS. In that test, blacks had higher TEWL levels than Caucasians, suggesting that in the pre-occluded state, blacks are more susceptible to irritation than Caucasians. The finding of this study contradicts the hypothesis that blacks are less reactive than Caucasians. Further, Wesley and Maibach observed that six out of eight studies demonstrated higher TEWL values in black skin (Table 1, item B) (18).

Gean et al. (18) found no statistically significant difference in the maximum LDV response between black and Caucasian subject groups when they challenged skin with topical methyl nicotinate (0.1, 0.3, and 1.0 M). Further, unlike the earlier studies, they found no difference in the blood flow and erythema responses between the two groups.

Guy et al. (15) supports the results finding that LDV measurements of induced blood flow after application of 100-mM methyl nicotinate reveal no significant differences between black and Caucasian subject groups; however, a significant difference was found using photoplethysmography (PPG). Caucasians had a greater PPG value than blacks did, suggesting that Caucasians may be more susceptible to irritation. The authors did not explain why blood flow measurements using PPG showed a statistically significant difference between the groups when LDV did not.

Berardesca and Maibach (16) also found decreased reactivity in blood vessels in the black test group than in the Caucasian test group. They measured the post-occlusive cutaneous

reactive hyperaemia—temporary increase in blood flow after vascular occlusion—after an application of a potent corticoid, and measured vasoconstriction using LDV; the black subject group had several significantly different parameters of the hyperaemic reaction. They found a decreased area under the LDV curve response, a decreased LDV peak response, and a decreased decay slope after peak blood flow, showing that blacks have a decreased level of irritation-induced reactivity of blood vessels. These results are consistent with their previous work.

In conclusion, older studies using erythema as the only indicator for irritation show that Blacks have less irritable skin than Caucasians, but more recent studies using objective bioengineering techniques suggest that the eye may have misled us to an incorrect interpretation.

ASIAN VS. CAUCASIAN IRRITATION RESPONSE

An early study comparing Caucasian and Japanese susceptibility to cutaneous irritation was conducted by Rapaport (28). He performed a standard 21-day patch test protocol on Caucasian and Japanese females in the Los Angeles area, in which 15 irritants (different types or concentrations of cleansers, sunscreen, and SLS) were tested. The results were reported according to the cumulative readings of all subjects in an ethnicity group for each irritant. Japanese women had higher cumulative irritation scores for 13 of the 15 irritants tested; Rapaport interpreted these findings to confirm the common impression that Japanese are more sensitive to irritants than Caucasians are. Also, this sensitivity was independent on the concentration or exact chemical formulation of the substance tested, suggesting that Japanese are in general more sensitive than Caucasians.

While these findings are important, it is difficult to interpret the data. First, as also noted by Robinson (12), Rapaport provides little experimental detail and data. For example, while the study required 21 separate days of irritation readings, only the end cumulative irritation scores are reported. Had he reported daily irritation readings, we would have been able to note the time pattern of response. Further, no statistical tests were conducted to ascertain if the differences between the Japanese and Caucasian subjects were statistically significant. Note, too, that the cumulative irritation test score does not distinguish between the intensity of a subject's response and the number of subjects responding. Thus it is possible, e.g., for a few extremely sensitive Japanese subjects to inflate the overall irritation score. Therefore, at the minimum, it would be helpful to provide standard deviations to rule out such problems.

What at first seems surprising, Basketter et al. (29) found that Germans are more sensitive than Chinese subjects. Subjects in Germany, China, and the United Kingdom were exposed to varying concentrations (0.1–20%) of sodium dodecyl sulfate (SDS) for four hours on the upper outer arm, and the resulting dose-response irritation was measured on the basis of erythema. They concluded that the German subjects tend to be more sensitive than the Chinese, and the Chinese to be slightly more sensitive than the British. This conclusion is the opposite of popular belief and of the Rapaport study, which indicated that Asians are more likely to develop ICD than are Caucasians.

There are, however, inherent flaws in this study, some of which the authors acknowledged. First and foremost, this study does not control the variables of time and location. The German and Chinese studies were performed in three to six weeks in the winter, while the U.K. study was spread over 15 months. Also, in particular, German winters are colder and drier than Chinese winters, which in turn tend to be colder than English winters. These variables will distort the results in a predictable way if we assume that an individual becomes more sensitive to ICD in colder and drier climates (2). We would then expect, on the basis of climatic conditions, that the German subjects would be more reactive than the Chinese, and the Chinese more reactive than the British. As these are the actual results, we cannot necessarily contribute the differences in irritant response to ethnicity, as it is reasonably likely that the differences are possibly due to weather conditions. Also, they mention that 15% of the U.K. volunteers were black. While they account for this by showing that the black irritant response was similar to the overall U.K. group response, it is scientifically problematic to mix racial groups in a study testing for racial differences. Furthermore, they supplied no statistical tests for their conclusion that Germans are slightly more sensitive than the other ethnic groups.

Table 2 Statistical Analysis of the Basketter et al. (29) study

	0.1% SDS	0.25% SDS	0.5% SDS	1.0% SDS	2.5% SDS	5.0% SDS	10% SDS	20% SDS
Germany	0.03	0.09	0.23	0.50	0.65	0.72	0.76	ND
China	0	0	0.01	0.21	0.45	0.61	0.79	0.90
U.K.	0.01	0.01	0.06	0.15	0.33	0.41	0.49	0.76
N	100	100	100	100	100	100	100	100
Z (Germany-China)	1.75	3.07*	4.79*	4.29*	2.84*	1.65	−0.51	NA
Z (U.K.-China)	1.00	1.00	1.92	−1.10	−1.74	−2.83*	−4.42*	−2.64*
Z (U.K.-Germany)	−1.01	−2.60*	−3.41*	−5.28*	−4.53*	−4.42*	−3.94*	NA

The numbers in the first 3 rows are the decimal value of the percentage of the group that developed a positive irritant reaction at a specific SDS concentration. The numbers in the last 3 rows are the *Z*-values. We applied the binomial test to ascertain the differences in the percentage response of the subject groups:

$$Z = \frac{r_1 - r_2}{[2r(1-r)/100]^{50}}$$

where r_1 and r_2 are the ratios for the 2 ethnic groups and r is the weighted average. Since the sample sizes for different groups are equal, r becomes the simple average. An asterisk indicates that the ratios are significant at the 5% level.

Note that all the U.K.-Germany differences, except 1, are statistically significant; however, more than half of the U.K.-China and almost half of Germany-China differences are not statistically significant. This indicates a larger statistically significant difference between the 2 Caucasian groups than that between the Caucasian and Asian groups.

Abbreviations: SDS, sodium dodecyl sulfate; Z, *Z*-values.

To shed more light on the results, we conducted simple binomial tests of the differences in the percentage response of the subject groups. Using the resulting statistics, we found a larger statistically significant difference between the two predominately Caucasian groups than between each of the Caucasian and the Chinese groups (Table 2). These results indicate that race may not be the predominant factor affecting susceptibility to ICD in this study; other uncontrolled variables may dominate the results.

Variables such as time and location were eliminated by the Goh and Chia (30) study that tested the susceptibility to acute irritant dermatitis in Chinese, Malay, and Indian subjects. These subjects were exposed to 2% SLS in the right scapular region, and resulting irritation was measured using TEWL. This technique is an objective way to indirectly quantify irritation—the higher the TEWL value, the greater the implicit irritation. There was no significant difference in the TEWL level of irritant skin in a three-way statistical test of the three racial groups. There was a significant difference, however, between the TEWL values of Chinese and Malay subjects so that Chinese subjects were more susceptible to contact dermatitis. While this test does not contribute to the discussion of the difference in predisposition of irritation in Caucasian skin versus Asian skin, it does add to the overall question of whether race can be a predisposition to irritant dermatitis.

Foy et al. (31) clearly added to our knowledge of the difference in the acute and cumulative irritation response in Japanese and Caucasian female skin. They reduced some variables that compromised other studies; location, time, season, and scores were the same for both study populations. Eleven different materials were tested in the acute test; they were applied to the upper arms for 24 hours, and irritation was measured on the basis of erythema. The cumulative test consisted of testing five irritants using a four-exposure cumulative patch protocol.

In the acute test, while there is a slight tendency toward greater susceptibility to irritation among Japanese subjects, only 4 out of the 11 irritants caused a significant difference in reactivity between the two groups—these were the most concentrated irritants used. This shows that perhaps for more concentrated irritants, there is indeed a statistical difference in the acute contact dermatitis response; of course, this study needs to be interpreted in context with others to follow. For the cumulative study, the skin irritation scores between the two test groups are close, but the Japanese tended to have slightly higher numbers. The differences, however, only reached statistical significance in two instances. And as the authors noted, it is

difficult to interpret the importance of those two instances since the statistical significant differences are not maintained at later points in the timeline. It is safe to conclude, therefore, that while the acute irritant response to highly concentrated irritants was significantly different between the Japanese and Caucasian subjects, the cumulative irritant response rarely reaches a statistical difference.

Studies that include both acute and cumulative irritant tests, like the one above, are more informative than single tests since they give a more complete view of differences in skin irritation between groups. Robinson (32) conducted a series of studies that tested racial differences in acute and cumulative skin irritation responses between Caucasian and Asian populations. In the first acute tests, Caucasian and Japanese groups were exposed on the upper outer arm to five irritants under occlusion for up to four hours. The resulting erythema was scored on an arbitrary visual scale. The results are represented as the cumulative percentage incidence of positive test reactions to the different irritants.

It is curious to note that while Japanese subjects tend to be more susceptible to acute irritation than Caucasians, neither one irritant nor one test time caused a significant response difference between the two groups. Further, note that for three of the five irritants only Caucasians reacted at early test times, contradicting the hypothesis that Japanese are more reactive to irritants. But even this trend-breaking difference is not considered statistically significant. The acute irritation response data were then reanalyzed in terms of possible differences in temporal response. The analysis showed that Japanese subjects generally react faster than their Caucasian counterparts, as indicated by their shorter TR50 values (the time it takes for the cumulative irritation score to reach 50%). While this result is interesting and adds the new dimension of temporal differences in reactivity between the two groups, hard data were not provided and statistical analysis was not conducted to see if this temporal pattern difference is indeed statistically significant.

The cumulative irritation test was conducted concurrently and on the same Japanese and Caucasian subjects. Four concentrations of SDS (0.025%, 0.05%, 0.1%, and 0.3%) were applied on the subjects' upper backs for 24 hours for 14 days. The resulting skin grades were summed for all subjects for all test days. For the two lower SDS concentrations the Japanese subjects reacted only slightly more than the Caucasian subjects, but only the difference in skin grades for 0.025% SDS reached statistical significance. When this data were analyzed in terms of temporal response, for the two lowest concentrations, the Japanese reacted only slightly faster than their Caucasian counterparts. Whether the difference in reaction time is statistically significant is not known.

In the same study, Robinson then applied both the acute and cumulative irritation protocols to compare three new subject groups—Chinese, Japanese, and Caucasian—with each other. The cumulative irritation study found no statistically significant differences between the different groups. In the acute test, he found that, in most cases, the Chinese subjects were more reactive to irritants than Caucasians, but that this difference significant in only one case, and he stated that most likely this was an anomaly. There was no discernable difference between the Japanese and Chinese groups. And surprisingly, when the Japanese subjects were again compared with the Caucasian subjects, as they were in the beginning of his study, the results showed no significant difference between the two groups.

While Robinson's first two-way irritation response comparison test between Japanese and Caucasian subjects did show some statistical differences, the fact that they could not be confirmed in the second half of the study emphasizes the difficulty in obtaining repeatable results in this type of study. For one, in the statistical sense, Robinson's sample sizes (approximately 20 people) were small, combined with the variability between human skin within an ethnic group; this makes it difficult to make concrete conclusions. His study showed, however, that there were essentially no significant differences between the Asian and Caucasian groups, at least none that could be repeated.

Robinson et al. (33) had similar results. Using the four-hour occlusion patch method, they compared the relative acute skin reactivity of Asian and Caucasian subjects using the irritation temporal response to measure the difference in reactivity between the test groups. They tested five chemicals, including 20% SDS and 100% decanol. Unlike the previously described study, they failed to find a statistical difference between the reactivity to multiple irritants between the two groups even at the four-hour mark. Then they did something new: They separated the

racial subpopulations into "sensitive" and "normal" groups to test any differences in percentage cumulative scores and temporal responses within these new groups but across race (i.e., he compared sensitive Asians with sensitive Caucasians). There were no statistically significant differences between subjects of the same skin type in different racial groups. This further contraindicates the hypothesis that Asians are more reactive to irritants than Caucasians.

Recently Robinson (34) compiled five years of his previous data and compared the acute reactivity differences between Caucasian and Asian (combined Japanese and Chinese) subgroups using the four-hour human patch method. The data were represented in terms of the time it took for subjects to have a positive response to the irritant chemical. Again, as in most experiments, Asians displayed a greater irritation response score than Caucasians. However, this difference only reached statistical difference at the four-hour mark, with SDS and decanol as the irritants. Note that while these results of this study are probably more representative of the population at large because of the relatively large sample size (200 plus), the data from this study were compiled from three different testing centers over five years. This could have potentially added uncontrolled and unaccounted-for variables.

In support of the long-held belief that Asians are more susceptible to ICD, several studies do indeed demonstrate this tendency (31,33,34). Rarely, however, is this trend statistically significant, and even more rarely can the statistical significance be repeated in another study. Therefore, it can be concluded from these studies that there is no fundamental difference between Asian and Caucasian cutaneous irritant reactivity—the overall irritant response and the time to reach that response is similar in both subgroups.

But the lack of comparable studies, small sample sizes, external variability, and intravariability within the subgroups make it difficult to completely dismiss Rapaport's original findings that Asians are more reactive than Caucasians. For example, different studies apply the irritant test material on different parts of the body, which might have different reaction responses. This makes it difficult to compare the results of one study with another and therefore raises the question of whether a more solid trend among studies would exist if the irritants were applied to the same anatomical site. Further, with regard to skin properties, Wesley and Maibach found that the data remain largely discordant and poorly characterized when discussing Asian skin (3). For the time being, however, in terms of topical product safety, risk assessment for occupational hazards, and global product marketing it would be practical to assume that few statistical differences between Asian and Caucasian cutaneous reactivity exist.

CONCLUSION

In Table 3 are summarized some potent factors that might influence the refinement of interpretation in future investigations. These studies demonstrate that there is little evidence of statistically significant differences in the irritant response between Caucasian and black or Asian groups. We can see no consensus on whether race is indeed an endogenous factor in ICD. Intuitively, we suspect that ethnic differences exist in skin function and may have evolved as have those in hair and other differences. Basically, the studies suggesting differences in skin (15,17) are "stress" in nature (pre-occluded). Presumably new insights into physiology, pharmacology, and toxicology may clarify this situation.

Also, it is possible that the well-known, divergent response to irritants is due to intraindividual variations in the skin irritation response (35–37). This is a relatively new idea, and therefore further studies need to be conducted in this area before a definitive statement

Table 3 Potent Factors that Might Influence Refinement of Interpretation in Future Investigations

Experimental design
Baseline versus "stress" test differences
Anatomic site
Open versus occluded irritant stresses
Ethnic groups in the same versus varying geography
Comparable climatic conditions
Presentation of hard data and statistical analysis

can be made linking intraindividual variation to ethnic differences in the intensity of an irritation response.

The above discussion is clearly limited in scope when considering the wide array of ethnic groups present globally. Considerable work remains to be done before the role of ethnicity in ICD is fully appreciated. Future investigations, such as that conducted by Peters et al. on Punjabi and Tamil subjects (38), is required before the interplay between skin and ethnicity can be completely defined.

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50 In Vitro Skin Irritation Testing on SkinEthic™-Reconstituted Human Epidermis: Reproducibility for 50 Chemicals Tested with Two Protocols

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INTRODUCTION

Evaluation of the irritancy potential to human skin of any chemical or formulation used in the chemical, pharmaceutical, and cosmetics industries is a necessity. Several in vivo and in vitro tests aim to determine the risk of irritation resulting from the contact between these compounds and human skin. The most commonly used test is the rabbit skin irritation test described in the OECD test guideline 404 and in the European Chemicals Bureau Annex V part B.4 (http://ecb.jrc.it/testing-methods/) and initially described by Draize et al. (1). This animal test consists in topically applying substances, which are raw materials or, depending on regulations, formulations (i.e., finished products), on the rabbit's shaved skin. A score is attributed according to physiological observations on the animals, which allows the classification of each tested product.

However, the Draize test presents several major disadvantages. The first is due to the fact that rabbit skin and human skin have different physiological properties and responses to environmental and chemical agents (2–5). Unfortunately, the biological basis for the variability of skin irritation among species remains unknown (6). Rabbit data have often been taken as reference to determine the irritant potential of chemicals although, to the exception of rare publications (2,4), few studies have compared data obtained both on animals and humans. Some compounds are more toxic for rabbits than for humans and vice versa (2,4,7). Moreover, the Draize test lacks reproducibility (8,9). The third major inconvenience concerns animal suffering and discomfort since eschar formation can be observed with severe irritants.

Few experiments were done on humans because of direct risk of lesion and intoxication for the subject, resulting from the application of potentially dangerous compounds. Among the available human data on chemical toxicity, some derive from chemical insults with severe irritants due to accidents at home or work or due to repeated skin exposure to moderate irritants. The other human data for skin irritation testing were obtained by patch testing performed on relatively high numbers of volunteers (4,10–16). Compounds were tested pure or diluted, for different application times, but the experiments were stopped when moderate to severe reactions to the test compound were observed. Many parameters influence the reproducibility of this type of tests. First, variability is observed in function of the patches used. York et al. (17) showed that generally the "Webril" and the "Hill Top" patches produced greater reactivity than the "Van der Bend" and the "Finn" patches. Some other parameters responsible for the variability of test results are directly correlated with the choice of the volunteers: the interindividual variability of reactivity is the principal factor (18). Moreover, interethnic differences have been observed (19,20). The reactivity of human skin changes also with the anatomical site (21) and decreases with age (20). Even abiotic factors must be considered since the seasonal variability plays a role in skin reactivity (22,23). The seasonal effect was particularly evident in the experiment described by Basketter et al. (18) when a four-hour patch test with SDS 20% provoked skin irritation in 45% of the volunteers in summer, but increased to 91% in winter.

The development of in vitro alternative methods for testing skin irritation has been the aim of an increasing number of scientists. This can be explained by their ethical advantage, and in several cases, also by their enhanced convenience. The skin irritation function test (SIFT) (24) and the pig ear test (25) are two of these in vitro methods. These tests are thus performed on ex vivo animal tissues (mouse and pig, respectively).

To permit the testing on human tissues without the disadvantages of performing tests directly on humans, the development of cell and tissue culture appeared promising. Several models are now commercially available for testing skin irritation (26–30). Using reconstructed epidermis, all classical methods determining cell viability (such as MTT reduction, resazurin reduction, LDH release) are easy to perform. Moreover, the SkinEthic™ model allows the measurement of additional endpoints such as the release of IL-1α and IL-8 (28,31–33). Reconstructed epidermis consists of human epidermal cells cultured in chemically defined medium by using semiautomatic production procedures, producing human epidermis standardized in terms of thickness, terminal differentiation, and reactivity to test compounds.

The repeated experiments described here are performed on different batches of human reconstructed epidermis. They correspond to different production cycles using epidermal cells of different donors. In vitro experimentation allows testing compounds on human tissues whatever the age, gender, and race of the donor. Each of the 50 chemicals has, at least, been tested in triplicate in two different experiments using two protocols: an in vitro patch test and a direct topical application test. The in vitro patch test protocol mimics closely the human in vivo patch test protocol (14). We applied the compounds on 0.95-cm^2 polypropylen Hill Top chambers® (Cincinnati, Ohio, U.S.) for four hours. The quantity of chemicals applied is proportional to the size of the patches (0.95 cm^2) used. This technique allows the containment of the product on a determined surface in the center of the 4 cm^2 epidermis. In parallel, our direct topical application test is performed by applying 100 μL of the test compound directly onto the epidermal surface of 0.63 cm^2 for four hours. Among the 50 chosen chemicals, 20 chemicals were previously tested in the ECVAM pre-validation study (PVS chemicals) on acute skin irritation (25) and 30 chemicals were previously tested in the in vivo human patch test (HPT) described by Basketter et al. (14) (HPT chemicals). After test compound application, tissues were incubated at 37°C, 5% CO_2 for four hours in both protocols. The 20 PVS chemicals were tested in two additional separate experiments using the direct topical application protocol (4 times in total). Multiple endpoint analysis including cell viability (MTT reduction), histology, and IL-1α release measurements was performed. Absence of direct interaction between test chemicals and the MTT solution or nonspecifically on frozen-killed tissues was verified. Our goal was to study the reproducibility of reference chemical testing on epidermis with two convenient protocols, and to compare the results with available in vitro, as well as animal and human in vivo data.

SKIN MODELS AND TESTS

Reconstituted Human Epidermis

Tissues (SkinEthic™, Nice, France) used were fully differentiated three-dimensional reconstituted human epidermal cultures grown on the air-liquid interface for 17 days in defined growth medium (27,34). Each experiment was performed in triplicate on one single tissue production batch, but different batches (different production cycles and/or donor cells) were used for each repeated experiment.

Selection and Coding of Test Chemicals

We chose reference chemicals upon two criteria: their irritation status should have been defined in the European Community classification, and, furthermore, they should have been tested either on other three-dimensional models (25) or by the human in vivo patch test (14). The present study includes irritant and nonirritant compounds. Details of the 50 chemicals tested are in Table 1. The experiment performed on run B was realized as a blind test. The 20 HPT chemicals were coded by H. Maibach, UCSF, United States.

Table 1 Chemicals tested and corresponding skin irritation data

	Compound	CAS no.	Supplier	EU class.	OECD class.	Human patch class.	S/L
1	Sodium lauryl sulphate (50%)	151-21-3	Sigma	I[a]	I[a]		L
2	1,1,1-Trichloroethane	71-55-6	Aldrich	I[a]	I[a]		L
3	Potassium hydroxide (5%)	1310-58-3	JT Baker	I[a]	I[a]		L
4	Heptanal	111-71-7	Aldrich	I[a]	I[a]		L
5	Methyl palmitate	112-39-0	Aldrich	I[a]/NC [b]	I[a]	NC[b]	L
6	Lilestralis / Lilial	80-54-6	Aroma & Fine Chemical	I[a]	I[a]		L
7	1-Bromopentane	110-53-2	Aldrich	I[a]	I[a]		L
8	dl-Citronellol	106-22-9	Aldrich	I[a]	SLI[a]		L
9	d-Limonene	5989-27-5	Aldrich	I[a]	SLI[a]		L
10	10-Undecenoic acid	112-38-9	Aldrich	I[a]	SLI[a]		L
11	Dimethyl disulphide	624-92-0	Lancaster	NI[a]	SLI[a]		L
12	Soap from 20/80 coconut oil/tallow		Quimasso	NI[a]	NI[a]		S
13	cis-Cyclooctene	931-87-3	Aldrich	NI[a]	SLI[a]		L
14	2-Methyl-4-phenyl-2-butanol	103-05-9	Aldrich	NI[a]	NI[a]		L
15	2,4-Xylidine	95-68-1	Aldrich	NI[a]	NI[a]		L
16	Hydroxycitronellal	107-75-5	Astier-Demarest-Leroux	NI[a]	NI[a]		L
17	3,3'-Dithiodipropionic acid	1119-62-6	Aldrich	NI[a]	NI[a]		S
18	4,4-Methylene bis-(2,6-ditert-butyl)phenol	118-82-1	Aldrich	NI[a]	NI[a]		S
19	4-Amino-1,2,4-triazole	584-13-4	Aldrich	NI[a]	NI[a]		S
20	3-Chloronitrobenzene	121-73-3	Aldrich	NI[a]	NI[a]		S
21	1-Decanol	112-30-1	Aldrich	R38[b]		NC[b]	L
22	2-Propanol	67-63-0	Aldrich	NC[b]		NC[b]	L
23	Isopropyl palmitate	142-91-6	Aldrich	NC[b]		NC[b]	L
24	Octanoic acid	124-07-2	Aldrich	R34[b]		R38[b]	L
25	Methyl caproate	106-70-7	Aldrich	NC[b]		NC[b]	L
26	Methyl laurate	111-82-0	Aldrich	R38[b]		NC[b]	L
27	Decanoic acid	334-48-5	Aldrich	R38[b]		R38[b]	L
28	Dodecanoic acid	143-07-7	Aldrich	R38[b]		NC[b]	L
29	N,N-Dimethyl-N-dodecyl aminobetaine (20%)		Albright & Wilson	R38[b]		R38[b]	L
30	Benzalkonium chloride (10%)	8001-54-5	Sigma	R38[b]		R38[b]	L
31	Dimethyl sulphoxide	67-68-5	Sigma	NC[b]		R38[b]	L
32	Polyethylene glycol 400	25322-68-3	Aldrich	NC[b]		NC[b]	L
33	Acetic acid (10%)	64-19-7	Sigma	R38[b]		NC[b]	L
34	Hydrochloric acid (10%)	7647-01-0	Prolabo	R38[b]		NC[b]	L
35	Sodium hydroxide (0.5%)	1310-73-2	Sigma	R38[b]		R38[b]	L
36	Heptanoic acid	111-14-8	Aldrich	R34[b]		R38[b]	L

(*Continued*)

Table 1 Chemicals tested and corresponding skin irritation data (*Continued*)

	Compound	CAS no.	Supplier	EU class.	OECD class.	Human patch class.	S/L
37	Lactic acid	50-21-5	Aldrich	NC[b]		R38[b]	L
38	Benzyl alcohol	100-51-6	Aldrich	NC[b]		NC[b]	L
39	Triethanolamine	102-71-6	Aldrich	NC[b]		NC[b]	L
40	Dodecanol	112-53-8	Aldrich	NC[b]		NC[b]	L
41	Tween 80	9005-65-6	Sigma	NC[b]		NC[b]	L
42	Benzalkonium chloride (7.5%)	8001-54-5	Sigma	R38[b]		R38[b]	L
43	Propylene glycol	4254-14-2	Fluka	NC[b]		NC[b]	L
44	Octanol	111-87-5	Aldrich	R38[b]		NC[b]	L
45	Eugenol	97-53-0	Sigma	R38[b]		NC[b]	L
46	Geraniol	106-24-1	Sigma	R38[b]		NC[b]	L
47	Linalyl acetate	115-95-7	Aldrich	R38[b]		NC[b]	L
48	Hexanol	111-27-3	Aldrich	R38[b]		NC[b]	L
49	α-Terpineol	10482-56-1	Aldrich	R38[b]		NC[b]	L
50	Ethanol	64-17-5	Merck	NC[b]		NC[b]	L

I = Irritant, NI = Non Irritant, SLI = Slight Irritant, S = Solid, L = Liquid.
[a]Fentem et al., 2001.
[b]Basketter et al., 1999.

In Vitro Direct Topical Test Protocol

Three reconstituted epidermal tissues of 0.63 cm^2 on 0.3-mL-defined maintenance medium in a 24-well plate were used per control or tested compound. Test compounds of 100 μL or 100 mg were homogeneously displayed on the total surface of the reconstructed epidermis. Negative controls and positive controls were run in parallel for each experiment. Cultures were incubated for four hours at 37°C, 5% CO_2. The three cultures were then transferred into new wells of the same 24-well plate containing 0.3 mL of maintenance medium. Tissues were washed three times with 0.5-mL saline solution A. With solids (powders or crystals), the insert was turned upside down before washing and—maintained in this position with forceps—knocked two- to threefold on the inner wall of a beaker to mechanically remove most of the applied compound. Histology, MTT reduction, and IL-1α release endpoints were measured as described below. Untreated tissues and H_2O-treated tissues were used as negative controls, while SDS 20% (14,25) and nonanoic acid–treated tissues (35) were used as positive controls. Negative controls were considered satisfactory if three criteria were met: a high cell viability measured by MTT reduction ($\geq$ 85% of untreated epidermis), a normal histology (score $\geq$ 75) (see histology scoring below), and no release of large amounts of IL-1α ($<$ 30 pg/mL). Positive controls were considered satisfactory when a low cell viability was measured by MTT reduction ($<$ 50%) and when a necrosed histology (score $<$ 75) and an increase of the amount of secreted IL-1α ($\geq$ 30 pg/mL) were observed.

In Vitro Patch Test Protocol

Three reconstituted epidermal tissues of 4 cm^2, placed on 1-mL-defined maintenance medium in a 6-well plate, were used per control or test compound. A measure of 75 μL of the compound was homogeneously displayed on a 0.95 cm^2 Hill Top chamber (Cincinnati, Ohio, U.S.), which was immediately applied, carefully, to the center of a 4 cm^2 culture. In case of solid compounds, 75 mg of the powder or crystals was spread on 0.95 cm^2 (same surface as for liquids) on the center of the culture and covered immediately by a Hill Top chamber. A 5-mm large brush was used to improve the contact between the compound/patch and the epidermal tissue. The patches were homogeneously applied with delicacy; strong pressure was avoided. Negative controls and positive controls were performed in parallel for each experiment. The Hill Top chamber was removed after a four-hour incubation at 37°C, 5% CO_2. No washing step was included in this protocol because most liquid compounds were absorbed by the patch. With solids, the culture was turned upside down, and—maintained in this position with forceps—knocked two- to threefold on the inner wall of a beaker to mechanically remove most of the applied compound. Histology, MTT reduction, and IL-1α release endpoints were performed as described below. Untreated tissues and H_2O-treated tissues were used as negative controls, while SDS 20% (14,25) and nonanoic acid–treated tissues (35) were used as positive controls. Negative controls were considered satisfactory if three criteria were met: a high cell viability measured by MTT reduction ($\geq$ 85% of untreated epidermis), a normal histology ($\geq$ 75) (see histology scoring below) and no release of large amounts of IL-1α ($<$ 105 pg/mL). Positive controls were considered satisfactory when a low cell viability was measured by MTT reduction ($<$ 50%) and when a necrosed histology ($<$ 75) and an increase of the amount of secreted IL-1α ($\geq$ 105 pg/mL) were observed.

Histology

Per test condition, and whatever the protocol, one of three tissues was harvested for histology. The tissues were fixed in a balanced 10% formalin solution and embedded in paraffin. Vertical sections measuring 4 μm were stained with hematoxylin/eosin and photographed under a microscope.

Scoring of histology sections was performed as follows:

- No or minor epidermal changes: 100
- Slight epidermal changes (stratum corneum thickening and/or dissociation and/or parakeratosis; slight edema and/or cellular alterations in the viable layers): 75
- Severe epidermal changes (marked edema and/or less viable cell layers and/or cellular alterations and/or partial tissue necrosis and/or partial tissue disintegration): 25
- Total tissue necrosis and/or tissue disintegration: 0

Cell Viability Measurement by MTT Reduction

The MTT test was used to measure the viability of living cells via mitochondrial dehydrogenase activity (36). The ring of 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), yellow, is cleaved by dehydrogenases, yielding blue/purple MTT crystals, which are insoluble in culture medium. An intense purple color is observed when the tissue is healthy, while the culture remains white when necrosis occurred.

Per test condition, the two remaining tissues were incubated in a 0.5 mg/mL MTT solution (0.3 mL MTT for 0.63 cm^2 cultures, and in 1 mL MTT for 4 cm^2 cultures) for a three-hour incubation at 37°C, 5% CO_2. MTT crystals of 0.63 cm^2 inserts were dissolved in 2 mL isopropanol. In the case of the 4 cm^2 inserts, a 0.5 cm^2 biopsy from the treated center of the culture was harvested using a 8-mm diameter biopsy punch (Stiefel) and plunged in 1 mL isopropanol. After an overnight extraction at room temperature, the quantification of cell viability was obtained by comparing the optical density of the extracts measured at 570 nm (reference filter 690 nm) in percentage to the negative H_2O-treated controls.

IL-1α Release

Conditioned media (3 per test condition) underneath the epidermal cultures were collected after the four-hour incubation with the chemicals and kept frozen at –20°C. Inflammatory mediator IL-1α was measured quantitatively using ELISA kits (R&D Systems, U.K., Catalogue number DLA50) (31,32). Results were expressed in picogram of mediator released per milliliter of conditioned medium.

Direct Interaction Between MTT and Chemicals

A measure of 100 μL or 100 mg of each compound was incubated in 1 mL MTT solution (0.5 mg/mL) for three hours at 37°C, 5% CO_2. The interaction was quantified by measuring the MTT/compound mixture OD value (200 μL in triplicate) at 570 nm (reference filter 690 nm).

MTT Interaction with Chemicals on Frozen-Killed Controls

The same procedure as for the direct topical application test protocol was applied onto frozen-killed tissues (–20°C, overnight). The results were expressed as the percentage of fold increase compared with the corresponding value of nontreated living control tissues.

Prediction Models

To classify the chemicals as irritants or nonirritants, we propose a prediction model based on the three endpoints described above. A chemical was classified as nonirritant when two or three of the endpoints led to the following results: cell viability measured by MTT reduction over 50% compared with that of the H_2O-treated control, normal histology (score $\geq$ 75), and a release of IL-1α comparable to that observed for the H_2O-treated control ($<$ 30 pg/mL for the direct topical application test and $\geq$ 105 pg/mL for the in vitro patch test).

On the contrary, a chemical was classified as irritant since two or three of the endpoints measured corresponded to the following criteria: cell viability lower than 50% compared with that of the H_2O-treated control, partial or total necrosis of the epidermal tissues (score $<$ 75), and an amount of secreted IL-1α higher than the IL-1α release induced by the H_2O-treated control ($\geq$ 30 pg/mL for the direct topical application test and $\geq$ 105 pg/mL for the in vitro patch test).

In parallel, a single endpoint prediction model (viability by MTT reduction only) was used for comparison.

Statistical Analysis

Specificity corresponds to the percentage of nonirritant chemicals (according to the EU classification) identified as nonirritants in our test.

Sensitivity represents the percentage of irritant chemicals (according to the EU classification) identified as irritants in our test.

Accuracy corresponds to the overall percentage of correct classification.

Pearson correlations, slope, and variation coefficient were calculated by Dr. Els Adriaens (University of Gent, Belgium).

RESULTS OF THE TESTS

MTT Interaction with Chemicals

Most of the compounds did not present significant interaction with MTT, nor directly, nor on frozen-killed controls. However, these two additional control experiments have shown that the interaction was significant for three chemicals (eugenol, potassium hydroxide 5%, and heptanal) for at least one of the two experiments. First, a direct contact between eugenol and MTT solution quickly produced a dark blue/purple color (OD = 1.4), while, in parallel, the MTT solution alone remained yellow (OD = 0.0). Moreover, eugenol on frozen-killed epidermal tissues induced the change of color from white to dark blue/purple after a four-hour incubation. The dissolution of the formazan blue crystals in isopropanol exhibited an OD value, which represented 82.2% of the OD value obtained for living untreated tissues (with the other 47 compounds, the epidermal tissues remained uncolored, and the OD values were negligible). Nevertheless, a strong direct interaction between a compound and MTT solution was not always correlated with an increased MTT value during the tests on living tissues or even on killed tissues. For example, 5% potassium hydroxide strongly reduced the MTT solution after direct contact, leading to a dark blue/purple mixture (OD = 1.6). On the contrary, 5% potassium hydroxide on frozen-killed tissues led to a light blue color of the culture. The corresponding OD value represented only 14.4% of the living untreated culture. Finally, in the case of heptanal, direct contact with the MTT solution provoked a change of color to blue (OD = 0.2). The application of heptanal on frozen-killed tissues led also to a blue color, with an OD value of 35% of the living untreated tissues.

In Vitro Direct Topical Test

Results of the multiple endpoint analysis of the repeated experiments are in Fig. 1 (PVS chemicals) and Fig. 2 (HPT chemicals). The percentage of cell viability (MTT reduction) is expressed in comparison to the H_2O-treated control. All controls of each experiment were satisfactory according to our criteria. Moreover, results obtained with the blind test were completely comparable to the normal test.

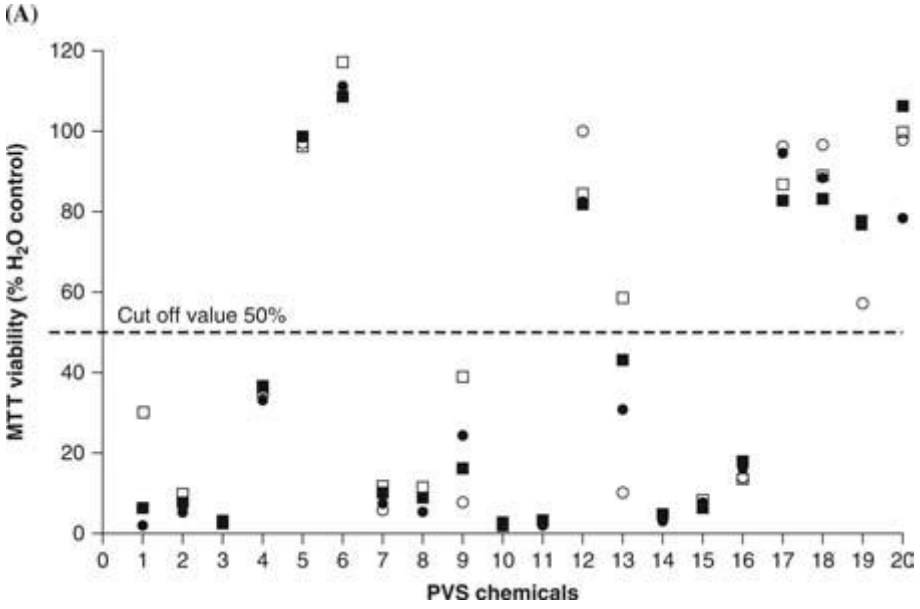

Figure 1 Multiple endpoint analysis for the PVS chemicals with the direct topical application test: (□) run D, (■) run E, (○) run G, (●) run H. (A) Viability (MTT reduction assay). (B) IL-1alpha release. (C) Histological observations. (See next page for Parts B and C.)

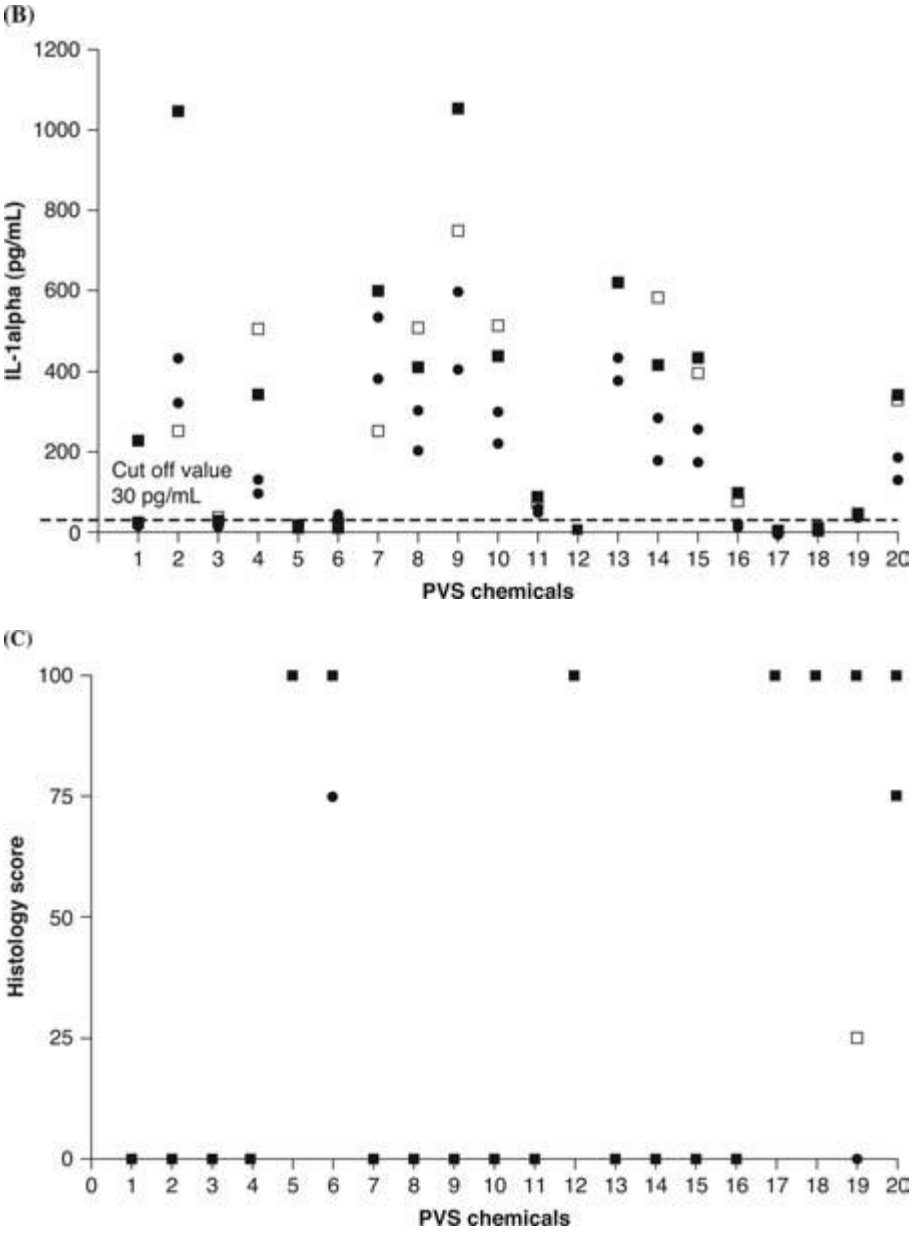

Figure 1 (*Continued*)

The response of the epidermal tissues to the chemicals could be classified in five families (Tables 2 and 3). The first is composed of compounds that induced a response comparable to the negative controls. The second family consists of the chemicals, which allowed a high cell viability, a normal histology (score $\geq$ 75), but provoked an increase of IL-1α release ($\geq$ 30 pg/mL). The third family is represented by chemicals, which allowed a cell viability higher than 50%, but necrosis was visible on corresponding histological sections (score $<$ 75), and an increase of the amount of IL-1α ($\geq$ 30 pg/mL) release was measured. The fourth family includes chemicals, which induced low cell viability, tissue necrosis (score $<$ 75), but no release of large amounts of IL-1α ($<$ 30 pg/mL). All remaining chemicals belong to the fifth and last family. This family is composed of the chemicals, which were responsible for a low tissue viability, tissue necrosis (score $<$ 75), and a significant increase of secreted IL-1α ($\geq$ 30 pg/mL). Whatever the family, we observed that in most cases the MTT values were of the same range for a given chemical. For example, among the 20 PVS chemicals, for which four independent experiments were

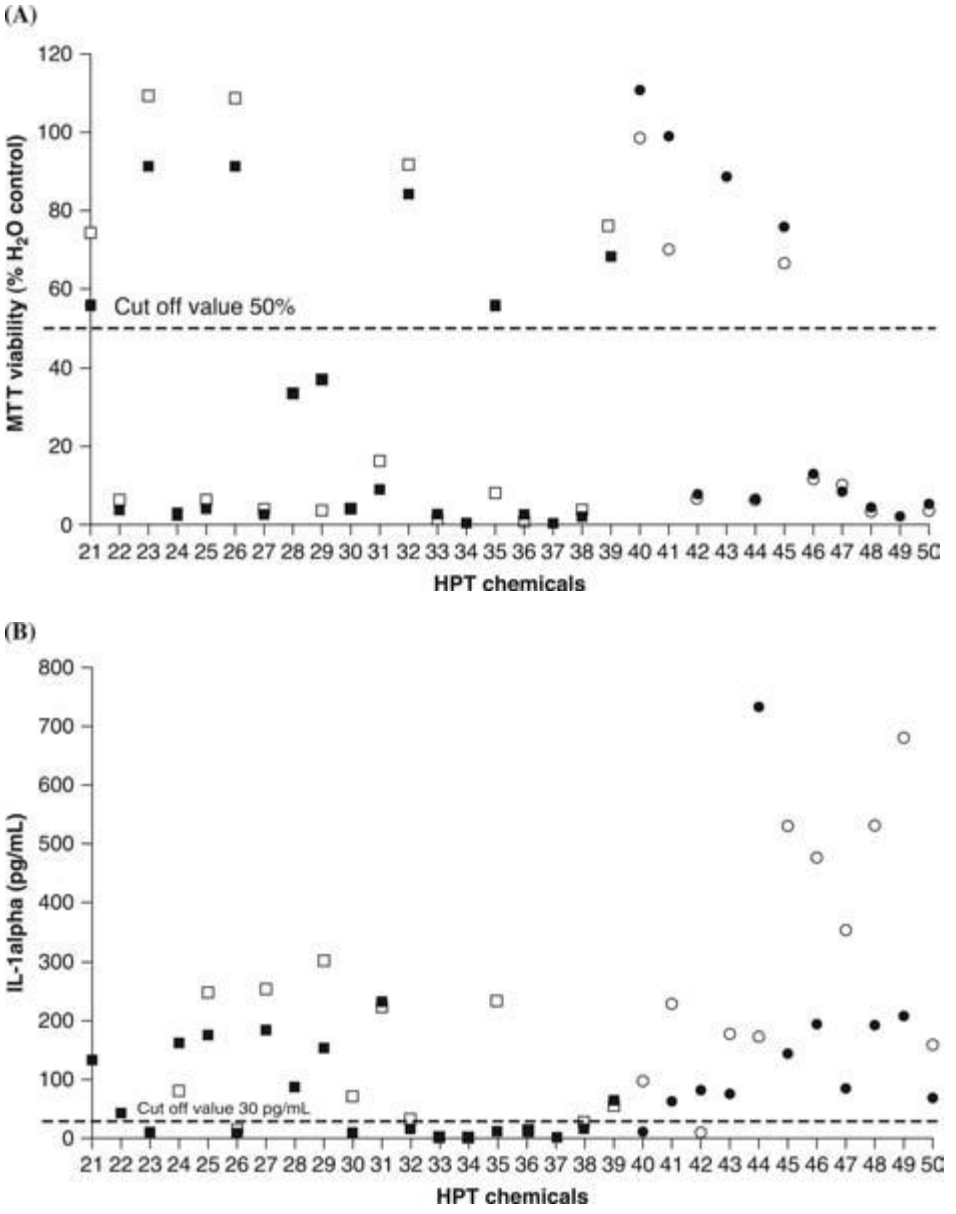

Figure 2 Multiple endpoint analysis for the HPT chemicals with the direct topical application test: (□) run A, (■) run B, (○) run F, (●) run I. (A) Viability (MTT reduction assay). (B) IL-1alpha release. (C) Histological observations. (See next page for Part C.)

performed, dimethyl disulfide–induced MTT values contained between 2.0% and 3.4% of negative control values; for heptanal they were contained between 33.1% and 36.0%, and for 3,3′-dithiodipropionic acid between 82.6% and 96.2%. Similarly, the histological appreciations were highly reproducible, especially when a given chemical was responsible for specific histological effects. Regarding the IL-1α release values, a reproducible effect was obtained. The chemicals induced two kinds of effects: on one hand, some compounds were responsible for an IL-1α release comparable to those of negative controls (e.g., methyl palmitate) and, on the other hand, some compounds provoked a significant increase of IL-1α release compared with negative controls ($\geq$ 30 pg/mL), although the absolute values presented high variations. For example, d-limonene provoked large amounts of released IL-1α (404.2–1055.9 pg/mL), and these amounts were higher than those of the 20% SDS-treated positive control tissues.

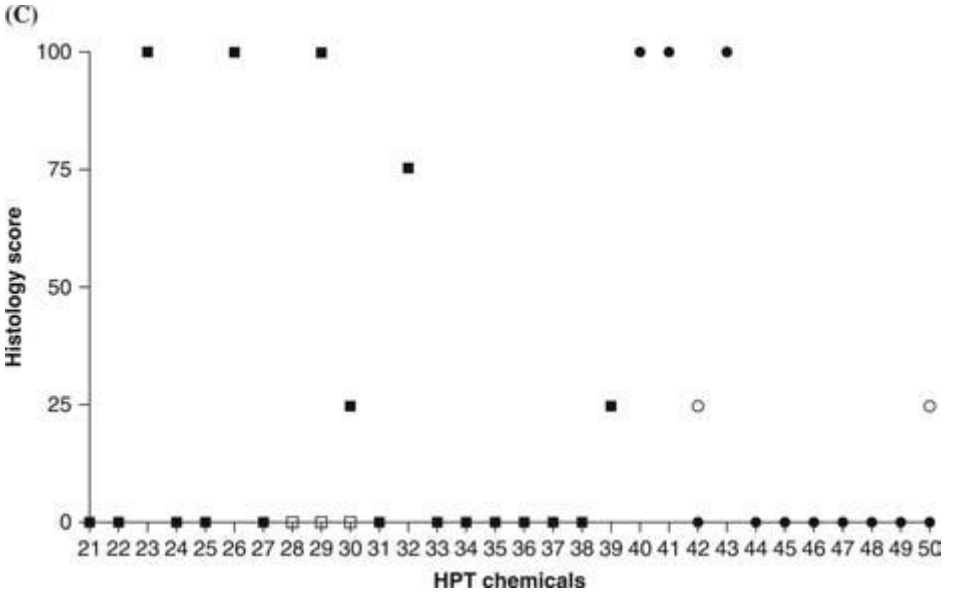

Figure 2 (*Continued*)

Table 2 Comparison of the results obtained with the 20 ECVAM chemicals

	Compound	EU class.	OECD class.	Epiderm class.[a]	Episkin class.[a]	SkinEthic dir. appl. (family) class.	SkinEthic patch (family) class.
1	Sodium lauryl sulphate (50%)	I[a]	I[a]	I	I	(4) I	(5) I
2	1,1,1-Trichloroethane	I[a]	I[a]	NI	I	(5) I	(5) I
3	Potassium hydroxide (5%)	I[a]	I[a]	I	I	(4) I	(5) I
4	Heptanal	I[a]	I[a]	I	I	(5) I	(5) I
5	Methyl palmitate	I[a]/NC[b]	I[a]	NI	NI	(1) NI	(1) NI
6	Lilestralis/Lilial	I[a]	I[a]	I	I	(2) NI	(1) NI
7	1-Bromopentane	I[a]	I[a]	I	I	(5) I	(3) I
8	dl-Citronellol	I[a]	SLI[a]	I	I	(5) I	(3) I
9	d-Limonene	I[a]	SLI[a]	I	I	(5) I	(3) I
10	10-Undecenoic acid	I[a]	SLI[a]	NI	NI	(5) I	(5) I
11	Dimethyl disulphide	NI[a]	SLI[a]	I	I	(5) I	(4) I
12	Soap from 20/80 coconut oil/ tallow	NI[a]	NI[a]	NI	NI	(1) NI	(1) NI
13	cis-Cyclooctene	NI[a]	SLI[a]	I	I	(5) I	(3) I
14	2-Methyl-4-phenyl-2-butanol	NI[a]	NI[a]	I	I	(5) I	(5) I
15	2,4-Xylidine	NI[a]	NI[a]	I	I	(5) I	(5) I
16	Hydroxycitronellal	NI[a]	NI[a]	I	I	(5) I	(6) NI
17	3,3'-Dithiodipropionic acid	NI[a]	NI[a]	NI	NI	(1) NI	(1) NI
18	4,4-Methylene bis-(2,6-ditert-butyl)phenol	NI[a]	NI[a]	NI	NI	(1) NI	(1) NI
19	4-Amino-1,2,4-triazole	NI[a]	NI[a]	NI	NI	(3) NI/I	(2) NI
20	3-Chloronitrobenzene	NI[a]	NI[a]	I	I	(2) NI	(1) NI

I = Irritant, NI = Non Irritant, SLI = Slight Irritant, NC = Non Classified.
Family 1: high cell viability, normal histology, no release of large amounts of IL-1α release.
Family 2: high cell viability, normal histology, increase of the amount of IL-1α.
Family 3: high cell viability, necrosed histology, increase of the amount of IL-1α.
Family 4: low cell viability, necrosed histology, no release of large amounts of IL-1α release.
Family 5: low cell viability, necrosed histology, increase of the amount of IL-1α.
Family 6: high cell viability, necrosed histology, no release of large amounts of IL-1α release.
[a]Fentem et al., 2001.
[b]Basketter et al., 1999.

Table 3 Comparison of the results obtained with the 30 chemicals tested in human patch test

	Compound	EU class.[a]	Human patch class.[a]	SkinEthic direct appl. (family) class.	SkinEthic patch (family) class.
21	1-Decanol	R38	NC	(3) I	(3) I
22	2-Propanol	NC	NC	(5) I	(6) NI
23	Isopropyl palmitate	NC	NC	(1) NI	(1) NI
24	Octanoic acid	R34	R38	(5) I	(5) I
25	Methyl caproate	NC	NC	(5) I	(5) I
26	Methyl laurate	R38	NC	(1) NI	(2) NI
27	Decanoic acid	R38	R38	(5) I	(4) I
28	Dodecanoic acid	R38	NC	(5) I	(3/6) I
29	N,N-dimethyl-N-dodecyl aminobetaine	R38	R38	(5) I	(1/3) NI / I
30	Benzalkonium chloride (10%)	R38	R38	(5) I	(5) I
31	Dimethyl sulphoxide	NC	R38	(5) I	(3/6) NI / I
32	Polyethylene glycol 400	NC	NC	(2) NI	(1) NI
33	Acetic acid (10%)	R38	NC	(4) I	(4) I
34	Hydrochloric acid (10%)	R38	NC	(4) I	(6) NI
35	Sodium hydroxide (0.5%)	R38	R38	(5) I	(1) NI
36	Heptanoic acid	R34	R38	(4) I	(5) I
37	Lactic acid	NC	R38	(4) I	(4) I
38	Benzyl alcohol	NC	NC	(4) I	(5) I
39	Triethanolamine	NC	NC	(3) I	(1) NI
40	Dodecanol	NC	NC	(2) NI	(1) NI
41	Tween 80	NC	NC	(2) NI	(1) NI
42	Benzalkonium chloride (7.5%)	R38	R38	(5) I	(5) I
43	Propylene glycol	NC	NC	(2) NI	(1) NI
44	Octanol	R38	NC	(5) I	(5) I
45	Eugenol	R38	NC	(3) I	(5) I
46	Geraniol	R38	NC	(5) I	(5) I
47	Linalyl acetate	R38	NC	(5) I	(3) I
48	Hexanol	R38	NC	(5) I	(5) I
49	α-Terpineol	R38	NC	(5) I	(5) I
50	Ethanol	NC	NC	(5) I	(1) NI

R38 / I = Irritant, NC = Non Classified, R34 = corrosive, NI = Non Irritant.
Family 1: high cell viability, normal histology, no release of large amounts of IL-1α release.
Family 2: high cell viability, normal histology, increase of the amount of IL-1α.
Family 3: high cell viability, necrosed histology, increase of the amount of IL-1α.
Family 4: low cell viability, necrosed histology, no release of large amounts of IL-1α release.
Family 5: low cell viability, necrosed histology, increase of the amount of IL-1α.
Family 6: high cell viability, necrosed histology, no release of large amounts of IL-1α release.
[a]Basketter et al., 1999.

According to our prediction of a model based on multiple endpoint analysis, the first and second families contained nonirritants, and the third, fourth, and fifth families contained irritants. The resulting classification is shown in Tables 2 and 3. A strong reproducibility was obtained between separate experiments. The one single exception for the 50 chemicals tested with this protocol was 4-amino-1,2,4-triazole (irritant in two and nonirritant in the two other experiments).

In Vitro Patch Test

Fig. 3 (PVS chemicals) and Fig. 4 (HPT chemicals) show the results obtained by multiple endpoint analysis with the in vitro patch test. As for the in vitro direct topical application test results (described above), both negative and positive controls were satisfactory. However, note that the amount of released IL-1α was four- to fivefold higher for the H_2O-treated control compared with that of the direct topical test. The ratio of medium volume to tissue surface was 0.48 mL/cm^2 for 0.63 cm^2 tissues, compared with 0.25 mL/cm^2 for 4 cm^2 tissues. Also, the topical application of an empty patch induces a slight increase of basal IL-1α secretion. We thus considered that the level of released IL-1α had increased significantly when it was over 105 pg/mL. Moreover, the cell viability of the positive controls of the patch test was

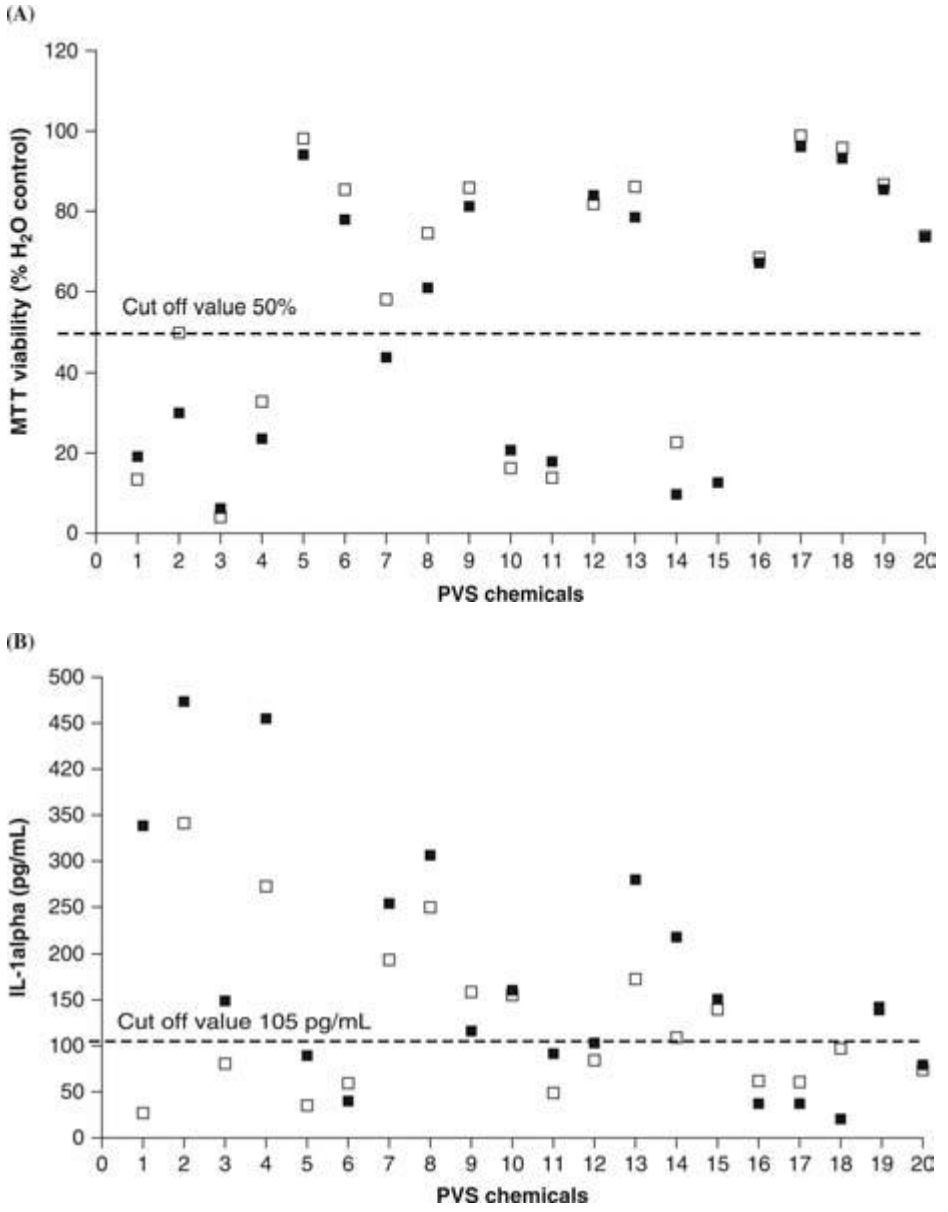

Figure 3 Multiple endpoint analysis for the PVS chemicals with the in vitro patch test: (□) run D, (■) run E. (A) Viability (MTT reduction assay). (B) IL-1alpha release. (C) Histological observations.

higher compared with those of the direct topical application test. More generally in this protocol, the percentage of cell viability was increased in most test conditions compared with the direct topical application protocol; this was probably due to the lower amounts (50%) of test chemicals, which, moreover, were applied to, and partially absorbed by the patches.

The same classification in families as for the direct topical test was applied to the experiments performed with this patch test protocol. However, a new sixth family has been created for hydroxycitronellal, 2-propanol, and 10% hydrochloric acid, which allowed a high cell viability and a small amount of IL-1α release, but provoked tissue necrosis as shown in histology sections.

The same prediction model as for the direct topical test was applied to the patch test results (Tables 2 and 3). A high intra-laboratory reproducibility could also be observed with this protocol to the exception of 20% *N*,*N*-dimethyl-*N*-dodecyl aminobetaine, dimethyl

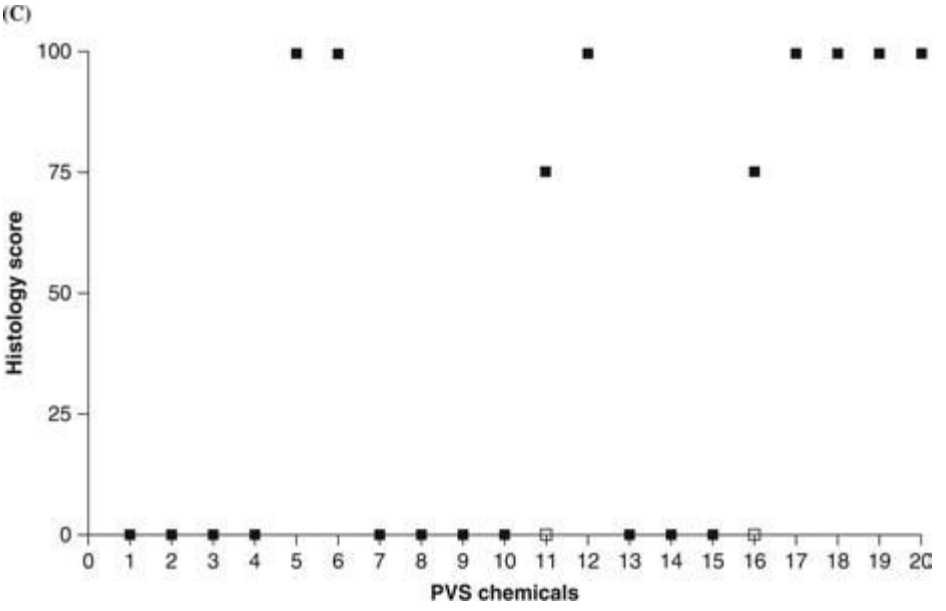

Figure 3 (*Continued*)

sulfoxide, and dodecanoic acid, which were classified as irritant in one experiment and as nonirritant in the other.

Comparison Between the Two Test Protocols

The comparison (Tables 2 and 3) between the predictions of the direct topical application test, and those of the in vitro patch test, shows that even when the experiments were performed using two protocols, the final results were similar for most chemicals. However, the direct topical application test seemed more sensitive, since hydroxycitronellal, 2-propanol, 20% *N*,*N*-dimethyl-*N*-dodecyl aminobetaine, 10% hydrochloric acid, 0.5% sodium hydroxide,

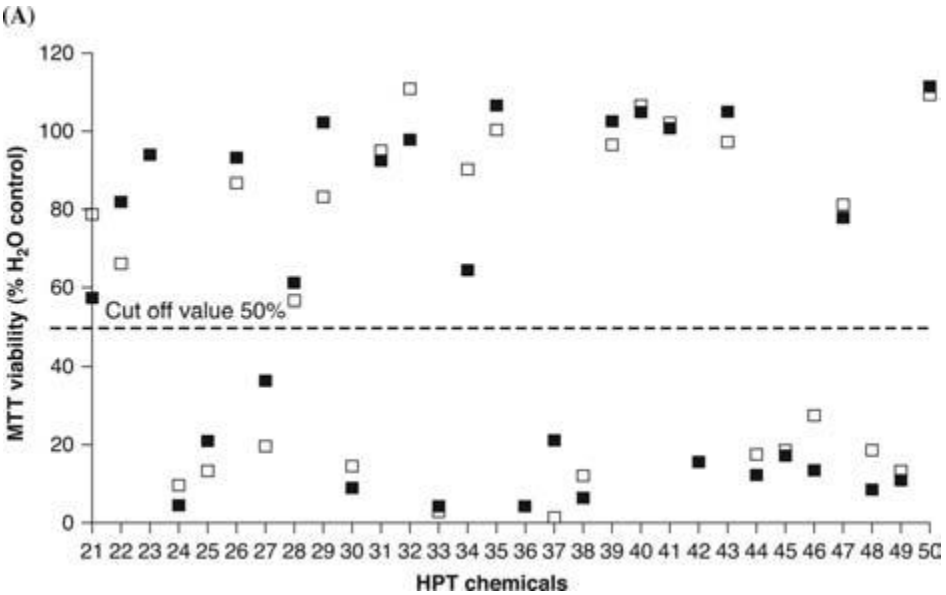

Figure 4 Multiple endpoint analysis for the HPT chemicals with the in vitro patch test: (□) run C, (■) run J. (A) Viability (MTT reduction assay). (B) IL-1alpha release. (C) Histological observations. (See next page for Parts B and C.)

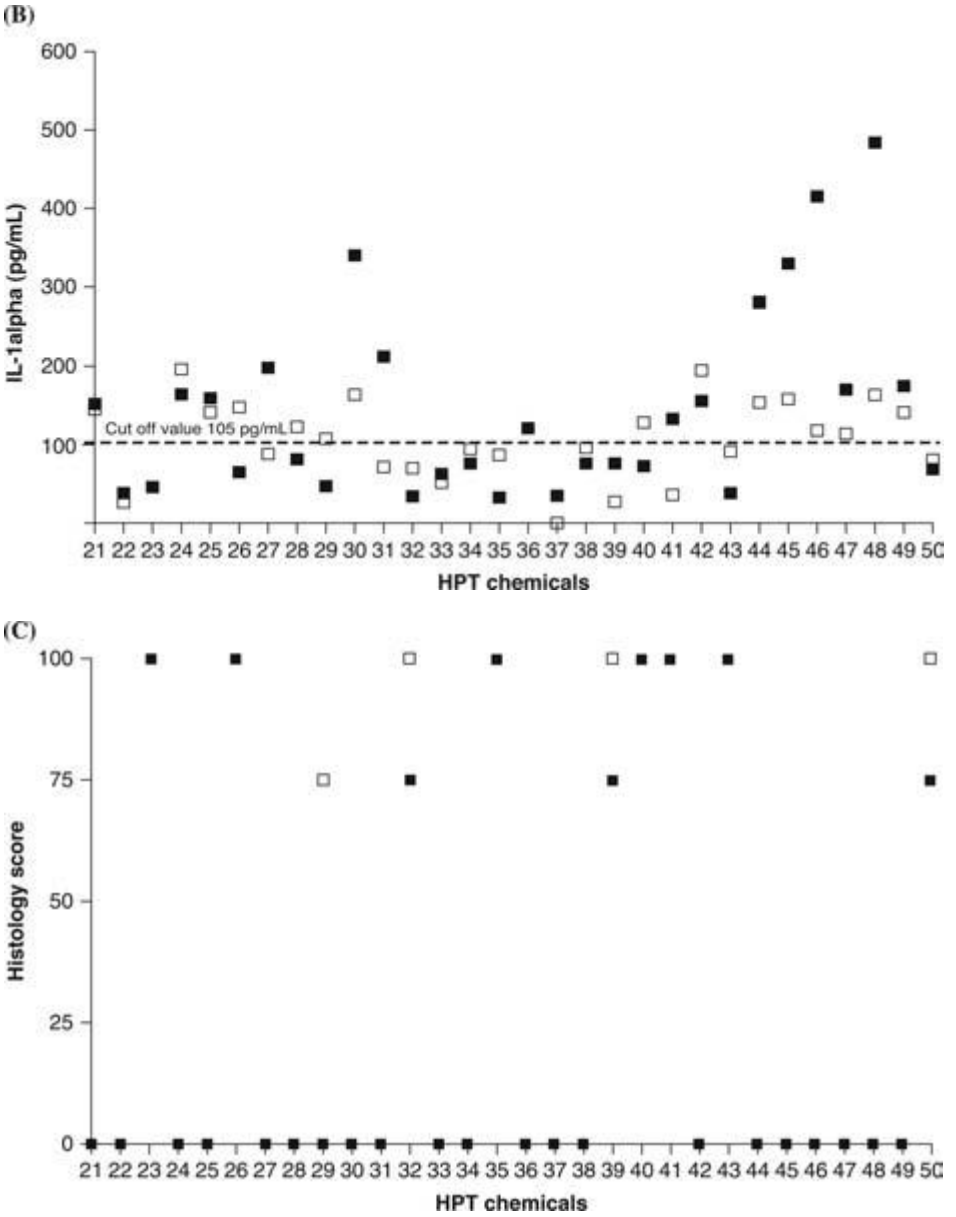

Figure 4 (*Continued*)

triethanolamine, and ethanol were detected as irritants with this protocol, while they appeared to be nonirritant with the in vitro patch test. These differences are discussed below.

Table 2 presents a summary of test results for the 20 PVS chemicals, including existing EU and OECD classifications, based on rabbit test results, as well as in vitro test results obtained with other tissue models [SkinEthic^{TM} direct topical application test, SkinEthic^{TM} in vitro patch test, Episkin^{TM}, Lyon, France, and EpiDerm^{TM}, MatTek Corp., Massachusetts, U.S. (25)]. All in vitro tissue models showed similar classifications of compounds whatever the tissue supplier. On the opposite, the comparison between the rabbit data and the human in vitro data revealed differences. In particular, this was true for the following compounds: dimethyl disulfide, cis-cyclooctene, 2-methyl-4-phenyl-2-butanol, and 2,4-xylidine. These compounds were classified as irritants by all the in vitro tests, and as nonirritants or slightly irritants according to the EU and OECD classifications.

Hydroxycitronellal was the one single PVS compound with an opposite classification using our two protocols: in the in vitro patch test, it was found nonirritant, like in the European and OECD classifications, while it was classified as irritant with the SkinEthic™ direct topical application test, similar to EpiDerm™ and Episkin™ classifications. Moreover, 4-amino-1,2,4-triazole (nonirritant in two experiments and irritant in the two others, with the topical application test) was classified unambiguously as nonirritant by the in vitro patch test.

A comparison between the EU classification, the human in vivo patch test, our in vitro patch test, and our in vitro direct topical application test is in Table 3. Among these 30 HPT chemicals, only 17 compounds were classified identically in the Draize test (EU classification) and in the human in vivo patch test (15). Among these 17 chemicals, 12 were also classified similarly with the in vitro direct topical application test; 13 were also classified similarly with the in vitro patch test; 10 of them are shared in common with the 12 cited above: isopropyl palmitate, octanoic acid, decanoic acid, benzalkonium chloride (10%), polyethylene glycol 400, heptanoic acid, dodecanol, tween 80, benzalkonium chloride (7.5%), and propylene glycol. Among the seven other chemicals (of the 17), methyl caproate and benzyl alcohol were found irritant with both in vitro protocols, while they were classified as nonirritant by the EU and OECD classifications. For the five remaining compounds, the in vitro direct topical application test led to the most severe classification, while 20% *N*,*N*-dimethyl-*N*-dodecyl aminobetaine was classified ambiguously, and 2-propanol, 0.5% sodium hydroxide, triethanolamine, and ethanol were classified as nonirritants with the in vitro patch test.

Among the 13 remaining chemicals, which were not classified similarly by the rabbit test and the human in vivo test, 12 compounds were found irritant with the in vitro direct topical application test. Thus, to the exception of the methyl laurate, which was classified as nonirritant, the in vitro topical application test also led to the most severe classification for these chemicals. In parallel, 10 of these 13 compounds were found irritant by the in vitro patch test, methyl laurate and 10% hydrochloric acid were classified as nonirritant, and dimethyl sulfoxide's classification was ambiguous.

Statistical Reproducibility

When the classification of a tested compound was unclear (I/NI), the most pessimistic prediction (irritant) was chosen, according to the principle of precaution.

Statistical analysis (including Pearson correlation and slope and variation coefficient) was performed for the different endpoints of both the direct topical application test and the in vitro patch test protocol. This study revealed an excellent reproducibility from one batch to another. Details are presented in Tables 4 and 5. Upon the batches D, E, G, and H, the Pearson correlation of MTT values of the repeated experiments was contained between 0.94 and 0.98, and the Pearson correlation of IL-1α values of the repeated experiments varied from 0.92 to 0.99.

The Pearson correlation was also calculated for the 50 compounds tested with both protocols. Concerning the direct topical application test, runs D and E were taken into account for the PVS chemicals. An MTT Pearson correlation of 0.96 was obtained for this protocol, and

Table 4 Correlations between MTT-values of repeated experiments

Pearson Correlation	D	E	G	H
D	1	0.98	0.94	0.97
E		1	0.97	0.98
G			1	0.98
H				1

Table 5 Correlations between IL-1α-values of repeated experiments

Pearson Correlation	D	E	G	H
D	1	0.92	0.92	0.94
E		1	0.92	0.93
G			1	0.99
H				1

Table 6 Summary of the results applied to both protocols for the 50 tested chemicals

	Dir. Top. Appl. Test	In vitro Patch Test
% Specificity	40.9	63.6
% Sensitivity	89.3	82.1
% Accuracy	68.0	74.0

the value was of 0.97 for the in vitro patch test protocol. The corresponding measures of variation (mean standard deviation) are 5.2% and 2.7%, respectively. In parallel, the IL-1α Pearson correlation for the 50 chemicals was evaluated as 0.75 and 0.65, respectively. Moreover, the mean standard deviation for IL-1α release corresponds to 4.3% of the mean nonanoic acid–treated positive control for the direct topical application test. For the in vitro patch test, the mean standard deviation value for IL-1α release is 15.6%.

The predictive capacity of both protocols for the testing of the 50 tested chemicals obtained in our laboratory is shown on Table 6. For the in vitro patch test particularly, multiple endpoint analysis allows an improvement of accuracy and sensitivity, but a decrease in specificity (specificity = 63.6%, sensitivity = 82.1%, and accuracy = 74.0%), compared with the single endpoint approach (MTT reduction only) (specificity = 72.7%, sensitivity = 55.6%, and accuracy = 64.0%). For the direct topical application test, multiple endpoint analysis allows an improvement of specificity, but a decrease in accuracy and sensitivity (specificity = 40.9%, sensitivity = 89.3%, and accuracy = 68.0%), compared with the single endpoint approach (MTT reduction only) (specificity = 50.0%, sensitivity = 82.2%, and accuracy = 68.0%).

VALIDITY OF THE EPIDERMIS MODEL TO PREDICT SKIN IRRITATION IN HUMANS

A multiple endpoint analysis, including percentage of cell viability (MTT reduction), histology, and IL-1α release, has been elaborated in an attempt to ensure the relevance and improve the quality of the test results. Among the families of compounds described above, the chemicals of the first family mimic the negative controls, and on the opposite, the chemicals of the fifth react like the positive controls. The classification of these chemicals as nonirritant or irritant, respectively, is therefore unambiguous since all three endpoints lead to the same conclusion. If all compounds reacted similarly, one single endpoint would have been enough to classify chemicals. However, a multiple endpoint analysis is not only a reassuring method, which permits multiple information, but it also reveals its usefulness for the chemicals of families two, three, and four. The second comprises chemicals, which allow high epidermal cell viability, a normal histology, but provoke an increase of IL-1α release. According to our prediction model, chemicals belonging to this second family are classified as nonirritants. However, the increase of epidermal IL-1α release could be an early sign of skin irritation. These compounds may be irritant over a prolonged or repeated application, or on weakened epidermis.

The chemicals of the third family provoke tissue necrosis, a higher amount of released IL-1α compared with H_2O-treated negative control; however, cell viability remains higher than 50%. This class contains, therefore, irritants. MTT reduction is an efficient cell viability test when the whole tissue is necrosed. Then, no or little mitochondrial activity is observed leading to a strong decrease of the amount of formazan blue crystals in comparison to the negative controls. However, when suprabasal cell layers are necrosed while the basal cell layer remains viable, a normal MTT reduction takes place, resulting in a percentage of cell viability comparable with negative controls (37). Moreover, some other false test results can be due to interactions between MTT and chemicals. This was the case for eugenol in the four repeated experiments on (living) cultures using the direct topical application protocol, the MTT values were contained between 66.5% and 75.9% of the H_2O-treated control, while histology showed necrosis, and the level of released IL-1α was high ($\geq$ 143.9 pg/mL). This elevated MTT value was due to the interaction between eugenol and the MTT solution, since the application of eugenol on killed cultures induces an OD value, which is 82.8% compared with the viable

untreated control tissue. On the opposite, we observed that the MTT values from eugenol-treated cultures in the patch test protocol are contained between 12.0% and 17.5% of the H_2O-treated control. This could seem paradoxal, but it is necessary to remember that a smaller quantity of compound is applied in this protocol and, moreover, that the patch partially absorbs the compound. In the case of eugenol, in patch testing, our hypothesis is that a four-hour incubation is sufficient to allow complete necrosis of the tissues and increased IL-1α release, but it did not allow eugenol to reach the MTT solution and interact with it. On the contrary, 5% potassium hydroxide did not provoke any interaction with the MTT, nor on living tissues nor on frozen-killed controls, although its direct contact with MTT solution provokes an OD value of 1.6. Concerning heptanal, the third and last compound, which showed interaction with MTT, MTT values of 35% of the H_2O-treated control were observed during the experiments on living tissues. This is completely comparable to the results obtained on frozen-killed controls. Although it is cautious to proceed to additional controls such as MTT interaction with chemicals, multiple endpoint analysis also allows detection of false viability measurements. Moreover, the classification according to our prediction model is not modified, even when the interaction with MTT is significant. In particular, it allowed to classify eugenol as irritant (with the direct topical application test protocol), while it would have been impossible to conclude with a single MTT endpoint approach.

The fourth family includes chemicals, which induced low cell viability and tissue necrosis, but did not provoke any significant increase of the IL-1α release. Therefore, most of the compounds of this class are highly irritant or corrosive. The small amount of released IL-1α could be due to the fast and massive destruction of the tissues, which did not have time to release cytokines. Another explanation could be the direct destruction of the cytokines by the chemical. Most of these compounds belong to the strong acid/base family. The epidermal tissues are severely damaged, and necrosis is provoked by these compounds. They can thus penetrate the epidermal tissue more easily and dissolve in the defined nutrient medium. This passive diffusion of the compound through the tissue to the medium was visually detected by the modification of the color of the medium.

Furthermore, some differences are observed between test results of the two in vitro protocols described. First, MTT values are in most cases higher with the patch test protocol. Accordingly, histology sections present a less severe necrosis. One possible reason for this is the reduced amount of chemical applied in the patch test in comparison with the direct topical test. A double quantity per centimeter square is applied on the tissues in this latter test. The quantity applied in the direct topical application test has been chosen to mimic published in vitro test protocols (25) and for its capability to cover uniformly the whole surface of the epidermis, whatever the texture of the compound to be tested. In the in vitro patch test, the quantity of chemical applied was defined proportionally to the human patch test described by Basketter et al. (14). Moreover, the structure of the Hill Top chamber itself is responsible for a partial absorption of some chemicals, reducing even more the amount of chemical that is in contact with the epidermis. It seems that the tissues necrosed more slowly in the case of irritant chemicals than in the direct topical application test. False negatives may result from these two parameters in the patch test protocol. In particular, in the case of the compounds of the sixth class, the increased MTT values may represent overestimations since high percentages of cell viability are observed, although the histology sections show necrosis.

However, although the chemical's effects seem to be less severe with the patch test protocol, the application of an empty patch alone is responsible for a four- to fivefold increase of the basal level of IL-1α release. This increase is probably due to the ratio of tissue surface to medium volume that was double for 0.63 cm^2 tissues compared with the 4 cm^2 tissues. Also, the topical application of an empty patch as well as some occlusive effects due to the patch induce a slight increase of basal IL-1α secretion.

The principal interest of in vitro experiments is not only to obtain reproducible data using more convenient and more ethical test protocols but also to produce useful indications on the human skin irritation potential of raw materials and finished products. The result of the comparison between in vitro and in vivo data is heterogeneous (Tables 2 and 3). Several chemicals were classified differently. On one hand, there are the compounds for which all in vitro and in vivo data corroborate, and, on the other hand, the chemicals for which in vitro classifications are in conflict with those obtained in vivo; or even more, chemicals for which in vivo rabbit data are in opposition to human in vivo data. Concerning the PVS chemicals, we

observed that our results resembled the results of those performed with other epidermal models in most cases. Notably, dimethyl disulfide, cis-cyclooctene, 2-methyl-4-phenyl-2-butanol, and 2,4-xylidine were classified irritant in vitro and nonirritant by the rabbit test. Lilestralis has been classified nonirritant with our two in vitro test protocols, although the EU and the OECD data filed it as an irritant. However, the documentation available from its suppliers does not mention any irritant properties for this compound, but only sensitizing effects. All results shown here are relative to the sample of chemicals obtained. Therefore, the comparisons made with in vivo and in vitro test results are only indicative, as other batches of chemicals were tested. Moreover, methyl palmitate, which was tested both on rabbits (25) and humans (14), is classified as irritant and nonirritant, respectively. All in vitro tests, like the in vivo patch test, classified this compound as nonirritant. Interspecies differences could be the explanation.

In vitro reconstructed human epidermal tissues mimic the biophysical properties of in vivo human epidermis. However, the reconstructed epidermis seems more sensitive to some families of compounds. Although 17 days of airlifted tissue cultures feature a fully differentiated stratum corneum (34,38), and a normal lipid composition (39), their barrier function seems to be less efficient (40,41) compared with adult skin samples, leading to a higher sensitivity to chemicals. This higher relative permeability may correspond to the epidermis of a newly (17 days) re-epidermized wound. This increased sensibility is considered as an advantage by Jones et al. (42) and Garcia et al. (41). Thus, when no toxicity is observed for a compound tested on reconstituted epidermis in vitro, the toxicologist can be confident about its safety regarding human use. Reconstituted epidermis could therefore be used according to the principle of precaution. Furthermore, in vitro tests using reconstructed epidermis present reproducible results leading to an unambiguous classification for almost all the tested chemicals.

Although the identification of the chemical's potential hazard is of the highest importance for both industries and consumers, its classification is difficult. Human data are certainly the most informative, but they are available for only few chemicals, and it is dependent on the protocol used, the age (20), the anatomical site (21), and the seasonal variability (18,22,23). On the contrary, animal data are more easily available, but the protocol used, the organism, and even the laboratory may provide different results as shown by Weil and Scala (8). Today, the relevance of the animal tests to assess human potential hazard is discussed, and the lack of reproducibility makes them even more questionable.

We cannot assure that our current protocols using three-dimensional reconstituted human epidermis is perfect to predict human skin irritation, but in addition to the classical advantages of in vitro methods, such as a great convenience and reduced costs, reproducibility is strongly increased compared with other methods. This reproducibility is not only seen for a given product on repeated experiments, but by individual endpoint measured for each tested compound. In our experiments using the PVS chemicals with the direct topical application protocol, the Pearson correlation is contained between 0.94 and 0.98. The MTT values show that they are almost always of the same magnitude, not just under or over 50% of viability. In the same way, we could make very similar histological observations for a chosen chemical. The statistical comparison of the IL-1α results shows that even if the amount of released IL-1α is not always of the same range for the irritant compounds (Pearson correlation for 50 compounds of 0.75 for the direct topical application test and of 0.65 for the in vitro patch test), chemicals can be classified in two classes. One contains the compounds, which always present an amount comparable to that of the negative control, and the other exhibits a significant increase of the amount of IL-1α, compared with the negative control. Such a reproducibility has never been shown with the Draize test or human patch test. Because of this strong reproducibility, the human in vitro epidermis already represents the tool of choice for screening compounds for their skin irritation potential.

Interestingly, note that the results obtained in our laboratory with the in vitro patch test protocol met the specificity, sensitivity, and overall accuracy performance criteria ($> 60\%$) defined for the ECVAM pre-validation study described by Fentem et al. (25) (Table 6). Moreover, a recent study performed by Kandárová et al. (43) revealed that dimethyl disulfide had been improperly tested in vivo. Consequently, the real classification of this compound is unknown. In parallel, methyl palmitate presents an ambiguous in vivo classification according to the literature (Table 1). If we remove these two chemicals, specificity obtained with the in

vitro patch test increases to 71.4%, sensitivity to 85.2%, and accuracy to 79.2%. Consequently, our in vitro patch test should be accepted for formal pre-validation by ECVAM.

However, all these test results are relative to the samples and lot numbers of the tested compound. We observed important variability in test results when certain compounds (lilestralis, hydroxycitronellal) came from different suppliers (unpublished data). For official validation studies, great care should be taken to control the quality of the reference compounds tested. Transferability being one of the parameters for ECVAM validation, it is indisputable that our encouraging intra-laboratory results should be followed by an inter-laboratory study. On the opposite, performance of the direct topical application protocol was disappointing compared with the in vitro patch test protocol and other published data. Taken together, this data set provides a platform for further mechanistic and validation studies. We do not wish to overgeneralize these data; judgment will continue to be required when extrapolating such information for new chemicals in terms of their complex uses in biology. Moreover, SkinEthic^{TM} epidermis is also involved in the current ECVAM skin irritation validation studies using the 15-minute direct application time followed by a washing step and a 42-hour incubation (44).

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51 Reconstructed Corneal and Skin Models

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INTRODUCTION

During the last decade tissue engineering became a progressing field in biotechnological research. The vision of medical treatment of burnt patients, the treatment of ulcera, and the idea of reconstructing damaged organs revealed very rapidly further possibilities. Tissues resembled not only morphologically the situation in vivo but also revealed comparable physiology. This made artificial tissues interesting for testing efficacy of pharmaceutical and cosmetics products. The development of the tissue models was paralleled by an increasing demand in using alternative methods for the identification of toxicological hazards inherent to raw material with the vision to replace animal testing for human safety assessment in toto.

Increasing efforts were made to validate such alternatives against the existing animal tests. Some of those are already successful, others, though promising, need further refinement.

Since the major field of applications for the cosmetic industry is doubtlessly the surface epithelial lining of humans, i.e., the skin and the mucous membranes of mouth and eye, progress in reconstructing such models is followed thoroughly and applications for these alternatives are evaluated extensively.

This chapter deals with the comparability of the reconstructed human corneal and skin models with the in vivo situation and shows up some areas of application in cosmetic science.

RECONSTRUCTED CORNEAL MODELS

Corneal Tissues

The epithelium of the eye surface distinguishes three regions: centrally the cornea, the limbus as transitional zone, and the peripheral conjunctiva. As a mucous membrane, it is a squamous epithelium, not keratinized but stratified. In contrast to the conjunctiva, the central cornea and the limbus are devoid of other cells such as Langerhans cells, melanocytes, and endothelial capillary cells.

The cornea itself is formed by three layers: an epithelium, a stroma, and an endothelial lining.

The epithelium consists of four to five cell layers with changing morphology. The cells of the basal layer are polygonal in shape. While proliferating, they produce lateral extensions to form so called wing cells. Apically they become flattened and compose a superficial cell lining. The outermost cells are strongly interlocked, building tight junctions to form a non-keratinized barrier. All cells contain a nucleus.

The stroma below the basal cells constitutes the majority of the corneal thickness. Directly below the basal cells of the epithelium, there is an acellular region called the Bowman's membrane, going over into the stroma. The stroma itself is a highly organized tissue made up by paralleled lamellae of collagen fibrils. Elongated fibroblast-like keratocytes are found throughout the stroma, running in parallel to the collagen lamellae. Posterior, a single layer of endothelial cells lines the stroma separated from it by the Descemet's membrane, a true basement membrane.

Damage to the Cornea

Irritation of the eye is a local and reversible response to external stimuli. Corneal and conjunctival cells are involved in this response. When evaluating damaging effects in animal testing like the rabbit Draize test, the majority derives from damage to the cornea (1). Studies on pathological changes after application of surfactants in standard animal irritation assays revealed that at early timepoints of three hours and one day after application, the potency of

the compounds could be differentiated. Innocuous and slight irritants affected superficial cells, mild to moderate compounds affected the epithelium and the superficial stroma, whereas severely irritating substances deteriorated deep stroma down to the endothelium (2).

Studies using non-surfactant compounds widened the insight into irritation, indicating that compounds may differentially injure cornea and conjunctivae. Furthermore, timepoints of evaluation played a critical role since damage at three hours could not predict severity of damage at one hour. Examples were given that, though in many cases damage by compounds is a progressive event first affecting the epithelium, some compounds have stronger effects on the stroma without involving the epithelium.

Thus, aspects of penetration, cytotoxicity, as well as time-related effects have to be considered when addressing hazard assessment by alternative methods. Therefore, as injury is a three-dimensional process, alternatives should focus on three-dimensional models.

Reconstructed Cornea Models

Corneal tissues produced by methods of tissue engineering resemble the in vivo situation more and more with respect to morphology, physiology, and biochemistry.

Tissue constructs exist on the basis of cells of different animal origin, i.e., human, rabbit, or bovine. They differ in their complexity. While some models are simply made up by epithelial cells (3), others comprise an outer epithelium grown on a stromal equivalent (4). The most complex equivalents even contain a posterior endothelial lining (5,6).

In all these models, the in vivo situation is closely mimicked. Depending on the origin of corneal cells, the epithelium is composed by 5 to 6 or 9 to 10 cell layers. Basal cells and wing cells might not be differentiated as clearly as in vivo, but stratification is obvious from flattened superficial cells being tightly packed and interlocked and joint by tight junctions forming a barrier. Slight differences in the morphology of this superficial cell lining are discussed to be due to the absence of lacrimal fluid and eyelid blinking (6). Basal layers express hemidesmosomes. Their internal placodes are connected to the cytoskeleton (3).

A functional expression of extracellular matrix plays an important role in the integrity and function of a tissue. A major component in the basement membrane, i.e., laminin, is detectable in the basal layers of the reconstructed skin model. In vivo fibronectin is localized in the basement membrane and promotes corneal migration and re-epithelialisation. In the reconstructed cornea models it is mostly detected at the epithelial-stromal junction as well as the collagen matrix making up the stromal equivalent (4,6). Integrins are expressed differentially throughout the epithelium according to their location of action. Their expression resembles the situation in vivo (4).

Permeation studies with pharmacologically active compounds underline the close resemblance and functionality of the described models. Permeation coefficients evaluated by penetration studies with organotypic cornea models differed form the in vivo situation in factors only smaller than two indicating the functionality of the epithelial barrier (6).

Use of Reconstructed Corneal Epithelia in Safety Assessment

For the prediction of irritating effects, mainly reconstructed epithelial models are used. In contrast to the more complex models, these are commercially available. The use comprises testing of pure compounds as well as formulations.

The main endpoint evaluated in such prediction models is cytotoxicity based on the conversion of MTT in the vital layers of the cornea (7,8). After application of cosmetic formulations for different time durations to the corneal equivalents, ET50 values are calculated estimating a time at which 50% of the tissues are mortal. On the basis of internal benchmarks, such models are used internally for product development (7). In a study with 68 tested products, irritant effects were overestimated by 10% compared with modified maximum average scores (MMAS) data, while there was only an underestimation of 1.5% (8). Therefore, it can be argued that they have a predictive ability to identify nonirritants from irritant products.

Testing pure compounds showed that prediction models from formulation testing with cytotoxicity as the only endpoint could not be transferred as such (8). Cytotoxicity testing focuses on the conversion of MTT in the viable layers of the equivalent not picking necrotic effects in the suprabasal layers. Additional parameters for evaluation of toxicity such as of histological parameters should be taken into account as well. A pre-validation study analyzing the viability of corneal tissues by the ability to reduce MTT after treatment with 20 pure chemicals, irritants and nonirritants, revealed an overall concordance of 80%. While all irritants

were predicted correctly (sensitivity = 100%), the prediction model produced a number of false positives (specificity = 56%) (9). Officially validated prediction models by European Center for the Validation of Alternative Methods (ECVAM) based on human reconstructed cornea do not exist yet.

Further endpoints such as release of cytokines and chemokines often used in other epithelial models are not subject to prediction models for eye irritation, though they are released after damage of the cornea (10), are detected in tears (11,12), and play a vital role in its regeneration (13). Studies have shown that cytokines can be produced after stimulation of corneal epithelial rat cell lines (14) and immortalized human cell lines (15). The evaluation of these parameters together with the already established multi-endpoint analyses might give us the chance to have a validated and internationally accepted alternative method to the Draize test.

RECONSTRUCTED SKIN EQUIVALENTS

Skin Equivalents

It has been 20 years since epidermal equivalents have been produced successfully for clinical applications (16). Since this time several equivalents of different complexity have been produced for scientific investigations as well as for commercial use. Today the most common equivalents are made up of keratinocytes. Biopsies from clinical surgery of adults and foreskin of young boys are sources of keratinocytes. In the simplest models, these cells are grown in culture under submerged condition for approximately 14 days until a multilayered tissue equivalent is formed. This equivalent is lifted to the so-called air-liquid interface in culture dishes to become stratified and cornified. Thus, such models distinguish several layers resembling in morphological characteristics to native skin: a stratum basale, a stratum spinosum, a stratum granulosum, and a stratum corneum.

Epidermal equivalents consist of 5 to 14 layers of viable cells depending on the model. Their thickness varies between 23 and 100 μm. Overall, they do not seem to be as thick as native skin with 80 to 90 μm.

The cells of the stratum corneum are of columnar to round shape. In contrast to native skin, they regularly contain intracellular lipid droplets. Keratinocytes of this layer are involved in the formation of the basement membrane. A lamina densa and a lamina lucida are produced by all equivalents in a patchy instead of continuous manner. Hemidesmosomes as anchoring structures between cells and basement membrane indicate the functionality of the stratum basale.

The stratum spinosum is characterized in the upper layers in vivo as in vitro by flattened cells. Unlike in native skin, few intracellular lipid droplets are recognized in cells of this layer.

The stratum granulosum produces numerous lamellar bodies of normal appearance thus correlating with the in vivo situation. Depending on the skin equivalent, they are more or less rapidly extruded at the interface to the stratum corneum. The unique organization of the alternating electron-dense and electron-lucent lipid lamellar sheets in the intercellular space between the corneocytes indicates a physiological processing of extracellular lipids of the stratum corneum.

The stratum corneum is made up of 14 to 25 cell layers compared with 15 to 20 in native skin. Its thickness ranges from 12 to 37 μm instead of 10 to 12 μm. In equivalents used for penetration studies, this number increases to 100 μm due to 100 layers of cells.

A detailed comparison of the characteristics of the different epidermal equivalents is given by Ponec et al. (17).

In concordance with the morphological resemblance of the reconstructed skin equivalents to native skin, expression and localization of differentiation markers correlate to the in vivo situation. Keratin 1 and 10 as indicators for early differentiation are present in all suprabasal layers of nearly all skin equivalents. The same holds true for loricrin and SPRR2, markers only present in the stratum granulosum. The localization of other differentiation markers like involucrin and transglutaminase does not resemble native skin as they are not restricted only to the stratum granulosum and found in all suprabasal layers. SKALP and SPRR3 are expressed in some epidermal tissue equivalents though absent in vivo.

Studies on comparative gene expression uncovered similarities between equivalents and native skin and showed up differences to monolayer cultures.

Monolayer cultures lack differentiation markers expressed in the upper layers of the epidermis, such as filaggrin, loricrin, involucrin, and keratins K1 and K10. Furthermore they

overexpress actin-associated cytoskeletal proteins and different integrins, reflecting their motility and adherence to the culture dishes, respectively. Proteins related to cell cycle and DNA replication are expressed in cultured keratinocyte monolayers while repressed in native skin. Together with a high expression of nucleoskeletal proteins, they reflect requirements in rapidly proliferating cells. The expression of corresponding genes identifies skin equivalents as metabolic active tissues somewhere in between monolayer cultures and native skin.

Similarities to native skin are found in the expression of cell-to-cell signalling molecules, as secreted proteins and cell surface receptors. This difference to monolayer cultures might indicate that cell-cell communication is important in the organization and maintenance of a stratified epidermis (18).

Recently, Poumay et al. (19) published a protocol that allows any experienced laboratory to produce its own epidermal equivalent. According to the protocol, one obtains a fully stratified epidermis within 14 days that reveals all the characteristic markers of differentiation (keratins 14 and 10, involucrin, and filaggrin).

Full-thickness skin models are of a higher complexity. They comprise a dermal and an epidermal compartment. Fibroblasts have to be cultivated in a dermal compartment and have to be given enough time to populate the space before keratinocytes can be seeded on top. Fibroblasts need a matrix that offers an environment facilitating the cells to exert their physiological characteristics. The use of collagen without any further treatment for its use as a dermal equivalent bears problems. The physiological characteristic of fibroblasts to exert traction forces leads to a contraction of the gel. Thus a matrix is required to be rigid enough to resist the contraction forces of the fibroblast while offering them the right physiological environment. Fibroblasts seeded to such a lattice produce their own extracellular material: collagen, elastin, fibrillin, fibronectin, and fibulin to mention a few. They organize the orientation of the fibers in the extracellular matrix as to be found in vivo. Short fibers are in close vicinity to the dermo-epidermal junction (DEJ) oriented perpendicularly, and long fibers oriented along with the DEJ in deeper parts of the dermis. This organization corresponds to the situation in vivo where similar differences can be seen between the papillary dermis and the reticular dermis. A proper DEJ is formed between the fibroblasts and the keratinocytes comprising the epidermis. Typical markers are expressed like laminin and collagen IV and VII. The basal layers of the epidermis express strong integrins, while in the suprabasal layers, one can find the differentiation markers transglutaminase and different cytokeratins (20).

Barrier Function and Penetration

One of the major functions of the human skin is the protection of the body against the loss of water. This function is fulfilled by a barrier in the upper layers of the stratum corneum being produced during the process of keratinization. In epidermal equivalents, this terminal differentiation is induced by culturing tissue equivalents at the air-liquid interface. Its development can be followed by the cutaneous permeability of caffeine in epidermal equivalents, which decreases with time at the air-liquid interface, finally reaching a plateau. After approximately 16 days in culture, no further improvement of the penetration characteristics can be observed (21).

The barrier is made up of three major components: the multiple lipid lamellae filling the extracellular space between the corneocytes, an impermeable cornified envelope made of proteins produced during terminal differentiation and coating corneocytes internally, and a corneocyte lipid envelope of ω-OH-ceramides, ω-OH-hydroxy acids, and free fatty acids situated externally to the cornified envelope. A proper composition and a structural organization of the lipids in the stratum corneum are required for a functional barrier (17,22). Though skin equivalents contain all major lipid classes, differences are noticed in content and profile either between the models or in native skin. None of the models resembled native skin in terms of lipid composition and ceramide profiling. With respect to ceramides, content of ceramide 2 is much higher in the epidermal equivalents, while polar ceramides are underrepresented or even missing.

Penetration studies with compounds of different lipophilicity revealed great differences with respect to flux across the membranes between excised human skin and epidermal equivalents. Permeability toward hydrophilic compounds as salicylic acid and caffeine showed increased fluxes by a factor of 20. Mannitol, another hydrophilic compound revealed an increased flux by factors of 20 to 50 depending on the equivalents tested (23). Hydrophobic

substances penetrated skin equivalents 900-fold faster. Reproducibility of penetration between different batches is regarded as an indicator for reproducible barrier function in skin equivalents (21,24). Depending on the compounds tested, one can conclude that reproducibility between batches is dependent on its lipophilicity. Gysler et al. (25) reported a variability of 14% between various batches regarding penetration of prednilosone being better than that of native skin. Garcia (21) demonstrated satisfactory coefficient of variation (CV) at approximately 20% regarding penetration of caffeine, confirming data published by Lotte et al. (24). Reproducibility of penetration of strongly hydrophilic mannitol was poor between batches in all the different models tested, while best for lipophilic lauric acid.

In a recent study by Schäfer-Korting et al. (26), different epidermal equivalents, SkinEthic®, EpiDerm®, and EpiSkin®, have been compared with human epidermis, bovine udder skin, and pigskin in a multicenter approach. Analytes were caffeine and testosterone. It turned out that the human reconstructed epithelia revealed inter-laboratory and intra-laboratory variability. In comparison with the human epidermis, the permeation of the compounds via the reconstructed epithelia are overestimated. Obvious differences were observed between the different equivalents. For testosterone, the model with the poorest barrier was SkinEthic, followed by EpiDerm and EpiSkin, for caffeine barrier was best in EpiDerm.

Thus, the barrier of commercially available epidermal equivalents is still less effective than that of native skin. This is discussed to be an intrinsic property of all epidermal equivalents (27). The different permeation characteristics between the reconstructed tissues indicate that though histological parameters are quite similar and resemble native skin, further research is necessary to reach a common standard achieved by every supplier. The only model resembling native skin in terms of composition and ceramide profile is the re-epithelialized deepidermized dermis by Ponec (28). With respect to penetration, this model shows best penetration characteristic for caffeine only differing to a factor of 2 in terms of flux from native skin (Ponec: personal communications).

Irritation Testing

The close resemblance of the epidermal equivalents to native human epidermis favors its use for the prediction of skin irritation that is still assessed on animals. Since keratinocytes are the first cells coming into contact with external compounds, they play an important role in the initiation and modulation of skin irritation (29). Markers produced and released by these cells are initial signals for visible clinical signs of irritation as edema and erythema, due to responses of the deeper tissues in human skin.

Early effects of irritation are mostly studied with models consisting only of keratinocytes, pure epidermis models. They are mostly commercially available: EpiDerm, EpiSkin, Apligraf®, and SkinEthic. Further models are in-house developments (30) or used for scientific purposes (31,32).

Recently, Poumay et al. (19) published a protocol that allows any experienced laboratory to produce its own epidermal equivalent. According to the protocol, one obtains a fully stratified epidermis within 14 days that reveals all the characteristic markers of differentiation (keratins 14 and 10, involucrin, and filaggrin).

In 2007, the ECVAM validated a prediction model to predict skin irritation with human reconstructed epidermal models. Analyzed were 58 chemicals, irritants and nonirritants. The assessed endpoints were viability measured by the turnover of MTT and analysis of released interleukin (IL) 1α to increase sensitivity. With the combination of the two endpoints, an overall sensitivity of 91% and a specificity of 79% for EpiSkin were achieved. It is regarded as a full replacement of the rabbit skin irritation test. For EpiDerm, a sensitivity of 57% and a specificity of 85% were obtained; analyzing IL 1α did not result in an improvement of these results. Thus, this system was recommended to be used in tiered testing strategy to assess skin irritation but cannot be regarded as a stand-alone prediction model (33). Catch-up validations of other commercially available systems (e.g., EST-1000®, Advanced Cell Systems®, and others) are undertaken so that several systems from different producers should be available soon to assess skin irritation in vitro.

An OECD guideline for the prediction of skin irritation in vitro does not exist yet.

Predicting potential irritation of ingredients is only one point that interests cosmetic industry. Another point is at least as important as the knowledge about hazardous effects in the predictivity of the irritating effects of formulations.

Measuring the turnover of MTT definitely faces the fact that the epidermal equivalents metabolize it only in the (supra)basal layers. Toxic effects not affecting the lower parts of the epidermal equivalent but the apical layers, as sodium lauryl sulfate (SLS), therefore cannot be predicted and evaluated (34). At least the evaluation of histological sections has to be taken into account. Studies therefore include in addition the determination of inflammatory mediators and enzyme release. Several studies dealt with the detection of inflammatory mediators, as cytokines, chemokines and prostaglandins. IL 1α, constitutively expressed, is one of the most important cytokines since it is released from keratinocytes immediately after membrane damage. Its release resembles data obtained by Lactate dehydrogenase (LDH) release. Further studies looked at IL 6. Though not released by pure epidermal equivalents, it is produced after irritation to model consisting of an additional dermal part (reviewed in 35).

IL 8 has strong chemotactic effects. It is induced by IL 1α and produced by keratinocytes and fibroblasts, therefore transducing effects to deeper parts of the skin. Prostaglandine E3 (PGE3) is the best investigated prostaglandin with respect to application of irritants to skin equivalents. Though some models produce PGE3 in a dose-dependent manner (32), other models fail to show such a relationship (35).

Perkins et al. (36) compared data concerning vitality (MTT), the release of enzymes as LDH and aspartate aminotransferase (AST), and the release of IL 1α after treatment of skin equivalents for definite times toward a human 14 days' repeated patch test, assessing the irritating potency of cosmetic formulation. The results revealed that for the prediction of irritating effects due to cosmetic formulations, endpoints like vitality (MTT) was useful for rank-ordering skin irritancy levels of surfactants. In addition with enzyme release (LDH and AST), these parameters distinguished lower and higher irritancy products. IL 1α was able to distinguish and rank-order the compounds of irritancy between these two extreme points. Another study compared the irritation effects of 22 cosmetic formulations. Endpoints measured in vitro were the determination of the effective time after application when 50% of the tissues lost their viability (ET50), percent of viability left 16 hours after application, the release of IL 1α, and the release of LDH. In vivo irritation was assessed under occlusive conditions by the modified Frosch–Kligman soap chamber patch test, in which the test material is applied repeatedly: the first time for 24 hours, followed by three applications of 6 hours on each of the following three days. Skin reactions are scored on each day until day 5. In addition, skin reddening was measured with a chromameter, and barrier interference was assessed by transepidermal water loss (TEWL) at the beginning and at the end of the study. The best rank correlation in the in vivo and in vitro data was achieved for ET50 followed by MTT at 16 hours and the IL 1α release, while for LDH release correlation was generally low.

Comparing the mean total score of the in vivo evaluation at day 5 with ET50, linear regression analysis gave coefficients of correlation of $r = 0.84$ to $r = 0.94$, depending on the model. Further analysis of the data by contingency tables taking into account a visual score of 2 as a cutoff value between irritancy and nonirritancy and MTT50 values as discriminator revealed equivalent results in all models tested: sensitivity = 92%, specificity = 100%, and concordance of 95%.

Protocols for the prediction of irritating effects of formulations are generally in-house methods. Therefore direct comparisons are hardly possible. Some of the protocols concentrate on the scoring and grading on biochemical parameters (Chatelain, personal communication), others use statistical methods to work out correlation between in vivo and in vitro data.

Generally one can conclude that in vitro assessment of irritation induced by formulations can distinguish between nonirritating and irritating formulations when data are compared with objective endpoint, clinical signs of irritation such as edema, erythema, and fissures. Other more subjective effects like stinging, itching, and pain are hardly to be predicted with the existing in vitro approaches.

Pharmacotoxicology

The reaction to topically applied irritants with the release and production of inflammatory mediators indicates that epidermal equivalents resemble native skin not only in terms of morphology but also in terms of physiology and biochemistry. Different studies deal with the biochemical characterization and metabolic competence of these models to identify them as alternatives for pharmacotoxicological studies. Gysler et al. (37) demonstrated the conversion of topically applied glucocorticoids into their metabolites by the metabolism of the

reconstructed skin models. The double ester prednicarbamate (PC) esterified at position 17 and 21 was hydrolyzed by esterases during its passage through the skin equivalent into the monoester P17EC and later on after passage nonenzymatically to P21EC. No PC itself was detected after passage, since it was totally metabolized. These results were in analogy to those obtained from experiments with native skin.

A fluorinated monoester betamethasone-17-valerate (BM17V) was not affected by esterases because of a missing ester bond at position 21, which passed the skin unconverted. The only metabolites found were BM17V and the nonenzymatically converted BM21V, after permeation of skin equivalents as well as native skin. Thus, the metabolism of PC and BM17V was well reflected by the skin equivalents.

Another study shows the applicability for screening skin-targeted androgen modulators since skin equivalents express type 1 5α reductase (5αR) activity. RT-PCR experiments revealed the expression of a unique 5α R1cDNA fragment, while there were no traces of 5α R2. This reflects the situation in native skin where 5α R1 is highly predominant and regarded as the important enzyme for testosterone turnover.

Topically applied testosterone is metabolized during permeation by 5α reductase mainly to dihydroxytestosterone. When inhibiting the enzyme by finasteride, this metabolic pathway is blocked dose dependently in skin equivalents as the main metabolite 4-androstene-3, 17-dione is produced (38).

In this respect, enzymes of the xenobiotic metabolism are of comparable importance. Activities of phase I enzyme cytochrome P 450 IA1 (CYP IA1) concerning its 7-ethoxyresorufin-O-deethylase (EROD) and 7-ethoxycoumarin-O-deethylase (ECOD) and of phase II enzyme glutathione S-transferase (GST) by 1,4-chlorodinitrobenzene (CDNB) conversion were examined in several skin equivalents (39). Furthermore, NAD(P)H:quinone reductase (NQR) activities were tested.

EROD activities were below detection levels in all tested models, but could be induced by 3-methylcholanthrene. This induction was strongly batch dependent in all the models, since some batches were not inducible at all. With respect to ECOD activity, there was a basal activity in all models. ECOD activity was only inducible in those batches that were inducible for EROD as well. Both activities could be inhibited by clotrimazole.

General GST activity against the standard substrate CDNB was detected in all equivalents. Variation within and between batches of all models did not exceed 20%. Activities were higher than in normal skin except for EpiSkin (40).

NQR is an enzyme that catalyzes the reduction of quinone, compounds present in the environment naturally or anthropogenic. Activities were tested against menadione. All tested models were competent concerning NQR, with SkinEthic showing highest and EpiDerm showing lowest activities. Activities in EpiDerm resembled those of native skin best. Inhibition by dicumarol could be induced in all the tested equivalents.

Studies on the gene expression of enyzmes of the xenobiotic metabolism in full-thickness models showed great resemblence to the in vivo situation. Full-thickness models were separated into dermis and epidermis, as well as native skin. Both compartments were analyzed for their gene expression to show differences in the expression pattern of epidermis and dermis and differences to the in vivo situtation. Phase I enzymes and phase II enzymes are comparably expressed in the different compartments of the native skin. Differences could be seen neither qualitatively nor quantitatively. Genes less strongly expressed could be induced by β-naphthoflavone (41). A review by Gibbs et al. (42) summarizes the actual status of xenobiotic metabolism in skin models compared with native skin.

These studies reveal the metabolic competence of skin equivalents, the physiological regulation of gene expression, and thus their use for pharmacotoxicological studies.

Studies with Melanocytes

For examination of mechanisms of skin tanning and the identification of ingredients influencing this process, the addition of melanocytes to epidermal equivalents is of great advantage. Cocultures of melanocytes and keratinocytes resulted in an enhanced survival of these cells and promoted melanin synthesis (43). Melanocytes and keratinocytes form together a so-called epidermal melanin unit. It typically consists of one melanocyte that is in contact with approximately 35 keratinocytes. Dendrites formed by melanocytes interdigitate into the intercellular spaces. Melanosomes produced within these dendrites are transported into the

keratinocytes. Here they orient themselves toward the nucleus and are organized in the form of an apical cap protecting the nucleus against irradiation. Thus functionality of the melanin unit can be monitored in the in vitro system. A recent study by Yoon et al. (44) shows the applicability of these in vitro systems to screen for melanogenesis affecting compounds. Reconstructed epidermal equivalents contained melanocytes of different origin: of African-Americans, Asians, and Caucasians. Two compounds, melanin-stimulating hormone (MSH) and dihydroxyphenyl alanine (DOPA), known as stimulators of melanogenesis, were examined for their ability to induce melanin content and tyrosinase activity. MSH increased tyrosinase activity in all three types of equivalent. This resulted in an increased content of melanin. In histological sections, Fontana–Masson staining of the melanin revealed an extended pigmentation in the upper layers of the skin.

DOPA increased melanin content, but decreased tyrosinase activity due to competition with the substrate used. Effects were more obvious in models with melanocytes from African-Americans and Asians than from Caucasians.

The key enzyme in the melanogenesis is the tyrosinase regulating the hydroxylation of tyrosine. Therefore known inhibitors affect the activity of this enzyme. Four inhibitors hydroquinone, arbutin, kojic acid, and niacinamid were tested. All compounds inhibited tyrosinase more or less dose dependently in all tissues, with hydroquinone having strongest effects. Melanin content was decreased in all tissues accordingly, whereas Fontana–Masson-stained section revealed a decreased melanin content only in the hydroquinone- and arbutin-treated equivalents.

Though some properties of reconstruced skin need further improvement, many characteristics resemble the in vivo situation. Besides a comparable morphology tissue, equivalents show similar reactions with respect to physiology, whether this is a biochemical answer to irritating compounds, the conversion of pharmacological active compounds by a comparable enzyme system, a resembling xenobiotic metabolism, or a coculture of skin relevant cell types.

Therefore tissue equivalents are used as a reliable tool supporting product development and since recently predicting the skin irritation potential of pure chemicals.

Further efforts are on the way to promote the acceptance of the use of these models also for regulatory acceptance.

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52 Seawater Salts: Effect on Inflammatory Skin Disease

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INTRODUCTION

Use of mineral spa water and seawater has been and continues to be a common treatment modality for inflammatory skin conditions such as psoriasis, atopic dermatitis, and irritant contact dermatitis. Spa water and seawater are noted for their relatively high concentrations of minerals such as strontium and selenium and for their high osmolarity relative to physiological saline (Table 1). Despite widespread use, few studies explore what aspect of seawater accounts for its therapeutic effect and what is its mechanism of action. Recent studies are summarized in Table 2 (1–4).

SEAWATER

Recent in vivo and in vitro studies lend credence to the common practice of applying seawater to inflamed skin. In acute eruptions of atopic dermatitis, seawater exhibited antipruritic effects as evaluated by a significant reduction of visual analogue scores for itching (1). In the setting of irritant contact dermatitis, Pacific ocean water compresses significantly decreased transepidermal water loss (TEWL) and increased skin capacitance compared with the deionized water control when the compresses were applied for 20 minutes at a time for several times over the course of two weeks (5). TEWL measures water barrier disruption, while capacitance measures stratum corneum water content. Thus, the results provide evidence for seawater's ability to inhibit skin barrier disruption and inhibit stratum corneum dryness in irritant contact dermatitis. Seawater has also been shown to be of benefit in psoriasis. In a randomized, double-blinded, controlled study, Dead Sea® salt baths, containing a high mineral composition, were administered daily at 35°C for 20 minutes for three weeks. Relative to the distilled water control, Dead Sea salt baths significantly decreased psoriasis area and severity index (PASI) scores in psoriasis vulgaris patients immediately after treatment, with therapeutic effects still significant one month after the treatment ended. However, there was no statistical difference in PASI scores and patient subjective evaluations between the treatment group that received Dead Sea salt baths and the group that received common salt baths [mostly sodium chloride (NaCl)] of the same osmolality. While this study supports seawater's therapeutic effects, it suggests that osmolality, instead of ion character, may act as the active component in seawater therapy (2).

CATIONS

Sodium

As a substantial component of seawater, sodium has been explored as an explanation for seawater's therapeutic effect. Similar to seawater, compresses with 500-mM NaCl alone have been shown to inhibit the increase in TEWL and increase skin capacitance and, thus, inhibit skin barrier disruption and inhibit stratum corneum dryness in irritant contact dermatitis (5). Sodium compresses for irritant contact dermatitis also decreased blood flow associated with irritation relative to baseline values when applied for 30 minutes twice a day for four consecutive days (6). However, the same study also showed that sodium compresses did not significantly change the skin's clinical appearance as measured by chromametry and clinical

Table 1 Composition of Seawater at 3.5% Salinity

Element	Atomic weight	Parts per million
Sodium (NaCl)	22.9898	10,800
Potassium	39.102	392
Magnesium	24.312	1290
Strontium	87.62	8.1
Selenium	78.96	0.0009
Bromine	79.909	67.3

Note: Parts per million = mg/L = 0.001 g/kg.
Source: Adapted from www.cea-life.com.

scoring. Sodium's role in seawater therapy is further questioned in this study by the finding that there was no significant difference between sodium compresses and cool water compresses, and the idea that neither osmolality nor ions but temperature explains the therapeutic effect of seawater (6). In vitro psoriasis studies also imply that sodium may not account for seawater's clinical value. Psoriasis is characterized by epidermal hyperplasia and heightened mitotic activity. In vitro, sodium salts failed to significantly affect fibroblast proliferation (3).

Potassium

As with sodium, studies yield conflicting results regarding potassium's role in explaining seawater therapy. The 10-mM KCl compresses inhibited skin barrier disruption by inhibiting an increase in TEWL but had no effect on capacitance, and thus, on stratum corneum dryness in irritant contact dermatitis (5). While potassium seems to have some effect on irritant contact dermatitis, its effect on psoriasis is less clear. An in vitro psoriasis experiment showed that potassium salts failed to significantly affect fibroblast proliferation (3), whereas another in vitro study that included two-hour incubations with salt solutions of 0, 50, 100, and 300 mM demonstrated that potassium salts were more effective than those of sodium and magnesium in reversibly inhibiting fibroblast proliferation and that KBr's inhibitory effect was similar to that of Dead Sea water (positive control) (4).

Magnesium

Recent studies suggest that magnesium has a greater influence on inflammatory processes in psoriasis than in irritant contact dermatitis. The 55-mM magnesium salt compresses showed no significant effects on barrier disruption or stratum corneum dryness in irritant contact dermatitis (5). However, magnesium illustrated significant and immediate (within 24 hours of treatment) inhibition of fibroblast proliferation in the in vitro psoriasis studies (3). Additional in vitro and in vivo studies showed that magnesium ions reduced the antigen-presenting capacity of Langerhans cells and are associated with reduced expression of HLA-DR and costimulatory B7 molecules by Langerhans cells (7). This last study hints at a possible role for magnesium in limiting the initial immune response or ongoing inflammation process in psoriasis.

Strontium and Selenium

Long suspected as the reason for the efficacy of Dead Sea water therapy, strontium and selenium have been shown to possess anti-inflammatory properties.

In a double-blind, vehicle-controlled, random-treatment study on irritant contact dermatitis, strontium salts were applied topically as pretreatment or mixed with irritant and were found to decrease the duration and magnitude of inflammation and sensory irritation (stinging, burning, and itching) without local anesthetic effects. Strontium inhibited total cumulative irritation from 56% to 81% according to a patient report, and these findings held true for the broad range of chemically unrelated irritants such as glycolic acid, lactic acid, aluminum chloride, and calcium thioglycolate that were used in the study (8). In another study, strontium exhibited anti-inflammatory effects on the molecular level.

An in vitro, controlled study looked at the effects of strontium and selenium on cutaneous inflammatory cytokines, IL-1α, IL-6, and TNF-α, at concentrations similar to those found in the Dead Sea. A weeklong continuous immersion of both healthy and atopic dermatitis skin in 260-mg/L strontium showed that strontium did not significantly affect cytokine levels in healthy skin. However, the study did show that strontium salts significantly

Table 2 Seawater and Its Effects on Inflammed Skin

Source	No. of patients	Type of dermatitis	Therapy	Duration	Evaluation	Significant results	Conclusion
Yoshizawa et al. (12)	3	Irritant dermatitis	Sea water (Pacific ocean)	20 min, 8× over 2 wk	TEWL, capacitance	↓TEWL, capacitance	Inhibits barrier disruption and stratum corneum dryness
			500 mM NaCl			↓TEWL, ↑capacitance	Inhibits barrier disruption and stratum corneum dryness
			10 mM KCl			↓TEWL	Inhibits barrier disruption only
			55 mM $MgCl_2$			Not significant	
			10 mM $CaCl_2$			Not significant	
Levin and Maibach (6)	9	Irritant dermatitis	H_2O compress	30 min, b.i.d. for 4 day	TEWL, LDF, chromametry, clinical score	↓TEWL, LDF	Both compresses *equally* inhibit barrier disruption and microcirculatory blood flow associated with inflammation but do not affect the clinical score or color
			Physiological saline compress			↓TEWL, LDF	
Celerier et al. (1)	In vitro	Atopic dermatitis	Spa water	1 wk continuous immersion	Inflammatory cytokine levels: IL-1α, IL-6, TNF-α	↓IL-1α, ↓↓↓IL-6, ↓TNF-α	Inhibits all three cytokines, but inhibits IL-6 to a greater degree
			260 μg/L $SrNO_3$			↓IL-1α, ↓↓↓IL-6, ↓↓TNF-α	Sr salts inhibit all three cytokines, but inhibit IL-6 to a greater degree. Sr salts selectively inhibit TNF-α production
			260 μg/L $SrCl_2$			↓IL-1α, ↓↓↓IL-6 ↓↓TNF-α	
			60 μg/L $SeCl_2$			↓IL-1α, ↓↓ IL-6, ↓TNF-α	Se salts inhibit all three cytokines, but inhibit IL-6 to a greater degree. Se salts selectively inhibit IL-1α production
			60 μg/L $SeNaO_3$			↓↓IL-1α, ↓↓↓ IL-6, ↓TNF-α	
			60 μg/L $SeNaO_4$			↓↓IL-1α, ↓↓↓ IL-6, ↓TNF-α	
Hiramatsu et al. (1)	20	Atopic dermatitis	Salt water	4 wk	VAS[a] for itching	5.05 ± 1.53% (pre) to 2.8 ± 2.4% (post)	Decreased itching of acute eruptions

(*Continued*)

Table 2 Seawater and Its Effects on Inflammed Skin (*Continued*)

Source	No. of patients	Type of dermatitis	Therapy	Duration	Evaluation	Significant results	Conclusion
Halevy et al. (2)	30	Psoriasis vulgaris	35°C Dead sea salt bath	20 min q.d. for 3 wk	PASI, patient-subjective evaluation	34.8 ± 24% reduction after 3 wk, 43.6 ± 31.1% reduction after 7 wk	Dead sea salt bath significantly decreased PASI over course of treatment; effect lasted for 1 mo after treatment. However, the Dead Sea salt bath's effect on PASI did not significantly differ from the common salt bath.
Gambichler et al. (2001)	10	Psoriasis	NaCl (24%) immersion followed by phototherapy (280–365 nm)	20 min, 30× over 7.5 wk	Clinical score (desquamation erythema, infiltration of plaques)	68.4% reduction from baseline	Significant decrease in clinical score from, baseline, but effect is, not significantly different from tap water control.
Levi-Schaffer et al. (3)	In vitro	Psoriasis	75 mM NaCl 75 mM KCl 75 mM $MgCl_2$ 75 mM $MgBr_2$ 75 mM KBr	1, 2, or 3 days	Fibroblast proliferation, cAMP levels	$MgCl_2$ inhibits proliferation by 50.7 ± 2.2%; $MgBr_2$ inhibits proliferation by 55.0 ± 2.3%; No treatment significantly affected cAMP levels	Mg salts had a significantly stronger inhibitory effect on fibroblast than other proliferation salt treatments of the same osmolarity. The inhibitory effect was immediate (within 24 hr)
Shani et al. (4)	In vitro	Psoriasis	0, 50, 100, 300 mM NaCl	2-hr incubation	Thymidine incorporation	Maximum reduction 44.4%	Br salts significantly inhibit growth greater than Cl salts. K exerted the greatest growth inhibition with KBr's inhibitory effect similar to that of the diluted Dead Sea brine.
			0, 50, 100, 300 mM NaBr			52.4%	
			0, 50, 100, 300 mM KCl			54.3%	
			0, 50, 100, 300 mM KBr			86.5%	
			0, 50, 100, 300 mM $MgCl_2$			32.5%	
			0, 50, 100, 300 mM $MgBr_2$			47.7%	

[a]Visual analog scale.

inhibited all three cytokines relative to baseline values, but that strontium selectively inhibited TNF-α to a greater degree (9).

A potential role for selenium in reducing inflammatory processes in skin has also been supported by recent studies. In the aforementioned study, healthy and atopic dermatitis skins were also immersed in 60-μg/L selenium solution for one week. In normal, healthy skin, selenium significantly decreased IL-1α cytokine levels but had no effect on IL-6 or TNF-α levels relative to that of the control medium. In atopic dermatitis skin, selenium salts significantly inhibited all three cytokines relative to baseline values but selectively inhibited IL-1α to a greater degree (9). Selenium has also been correlated with the duration and severity of psoriasis and may be related to the protective function of selenoproteins (thioredoxin reductases and glutathione peroxidases) against ultraviolet-induced cell damage and death. Both low plasma selenium and low plasma glutathione peroxidase activity have been seen in psoriasis patients. Patients with a longer history (>3 years) of psoriasis exhibited a significantly lower selenium level compared with patients with a shorter history of psoriasis (<10 months). Selenium also seemed to correlate with the severity of psoriasis in this study. A significant inverse relationship was found between RBC glutathione peroxidase and psoriasis area and severity index scores in individuals with psoriasis of greater than three years (10).

ANIONS

Bromine

Of the few anions in seawater that are studied, bromine affects skin disease processes the most. In an in vitro study of psoriasis with two-hour incubation with Dead Sea brine, NaCl, NaBr, KCl, KBr, MgCl, MgBr at 0, 50, 100, and 300 mM, bromide salts significantly inhibited fibroblast proliferation as compared with chloride salts. When combined with potassium in KBr, bromine's inhibitory effect was similar to that of diluted Dead Sea (positive control) (4). In another in vitro study, magnesium bromide inhibited fibroblast proliferation to a greater extent than magnesium chloride (55.0 + 2.3% vs. 50.7 + 2.2%), lending further evidence that if anions play a part in seawater therapy, bromine, not chlorine, is most likely to be the active anion (3).

With a paucity of studies and small sample sizes in each study, it is difficult to definitively say whether seawater or its individual components offer any clinical benefit in the inflammations of the skin. If seawater is proven to have therapeutic value, further studies will be needed to explore whether it is the synergism between seawater's various components, the osmolality, or individual ions alone that mediate its effect. As inflammatory skin diseases like eczema and psoriasis are accompanied by a defect in permeability barrier function, improving barrier function results in reduced inflammation. Thus, possible mechanisms of action of seawater salts include putative effects on barrier functions. This effect of ions on barrier function has been studied extensively (11,12). Currently, Dead Sea salt is sold in many countries and is used in clinical treatments and private bathtubs, but in much lower concentrations than the Dead Sea itself. Recent interest in the science of alternative medicines may be a stimulus for a more complete biological analysis of these ancient practices.

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53 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 suspected 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 that 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 a dermatologist verifies it.

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 cosmetic allergen is by no means a simple task. It demands special skills and interest on the part of the dermatologist, although labeling of all cosmetic ingredients 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 the frequently used products to cause more skin reactions than the more exclusive products, simply because more people are exposed to the former. 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 the simplicity of formula. The fewer the constituents, the easier it is to identify the offending substance should difficulties arise, and there would be a lesser danger of synergism. The presence of more ingredients leads to an increase in the chance of the skin's sensitization to 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 needed for water-based or other easily contaminated products are the 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 methyl (chloro)isothia-zolinone illustrate this problem very well. At first, a 50-ppm concentration of this agent was allowed for use in cosmetic products in the European community, following which 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 about 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. A more or less strict quality control of raw materials and finished products has long been the general practice in modern cosmetic manufacturing. However, one can never rule out the sensitizing potential of impurities in these materials (5).

Common Use of Cosmetic Ingredients in Pharmaceuticals

Patients easily become sensitized to topical pharmaceutical products, which, unlike cosmetics, are most often used on a diseased skin. However, once sensitization has occurred, they may react also to the 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, and applies to all other cosmetic ingredients as well.

Penetration-Enhancing Substances

The chemical environment can substantially affect a person's 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 face care product, along with hair products may cause an allergic reaction only on the eyelids. Moreover, "ectopic dermatitis" [caused by the transfer of an allergen by hand, as often occurs with tosylamide/formaldehyde (i.e., 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 shared between partners) (8) often occur only on "sensitive" skin areas such as the eyelids, lips, and neck.

Moreover, the penetration potential of cosmetics is heightened in certain "occluded" areas, such as the body folds (axillary, inguinal) and the anogenital region, because of which the risk of contact sensitization is increased. 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 the 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 a 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 body care products used to alleviate dry, atopic skin and with barrier creams used 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 body care 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 or several-times-a-day usage 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 products, e.g., day and night creams, foundations, and cleansing products, because a manufacturer will often use the same preservative system for all his 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 by 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 the 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, well-circumscribed patches that appear in areas where dabbing-on perfumes are used (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 been shown to contain well-known allergens such as cinnamal (cinnamic aldehyde) and isoeugenol (18).

As reported in the literature, the fragrance mix remains the best screening agent for contact allergy caused by perfumes, because it would detect some 70% to 80 % of all perfume allergies (19,20). However, it depicts also the need to test with additional perfume allergens.

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 skin care products (creams, lotions, masks), sunscreen products, makeup (foundations, blushes, powders), cleansers (lotions, emulsions), and cosmetic appliances (sponges), perfumed products (aftershave lotion)
Forehead	Hair care 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, hair care products
Head	Hair care products (hair dyes, permanent-wave solutions, bleaches, shampoo ingredients), cosmetic appliances (metal combs, hairpins)
Ears	Hair care products, perfume
Trunk/upper chest, arms, wrists	Body care products, sunscreens, and self-tanning products, (elbow flexures) cleansers, depilatories
Axillae	Deodorants, antiperspirants, depilatories
Anogenital areas	Deodorants, moist toilet paper, perfumed pads, depilatories
Hands	Hand care products, barrier creams, all cosmetic products that come in contact with the hands
Feet	Foot care products, antiperspirants

Indeed, testing with additional markers, for example, the individual components such as hydroxyisohexyl 3-cyclohexene carboxaldehyde (Lyral®), farnesol, and citral, as well as with the complex natural mixtures (21–25), increases the sensitivity of the testing. Because of the increasing importance of fragrance allergy and to ensure that sensitized consumers are adequately informed, 26 fragrance components are labeled as cosmetic ingredients on the package [Annex 3 (Table 2 of the cosmetic irective (26)]. With fragrance, allergy-associated positive patch test reactions frequently occur and often indicate the presence of common or cross-reacting ingredients in natural products, the occurrence of cross-reactions between simple fragrance chemicals, or concomitant sensitivity. Moreover, oxidation products of fragrance ingredients, such as limonene (27) or resin acids (being the main allergens in colophony), found as contaminants in tree moss (a widely used substitute for oak moss) as well as in oak moss itself (28), play an important role in the allergenic potential of these substances (29).

Preservatives

Among the allergy-causing agents, preservatives are second in frequency to fragrance ingredients; they are important allergens in cleansers, skin care products, and makeup (2,30). However, within this class, important shifts have occurred over the years (30,31).

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 methyldibromo glutaronitrile—as used in a mixture with phenoxyethanol, better known as Euxyl K400—did gain importance in this regard (12,30–34), although the frequency of positive reactions observed seems to be influenced by the patch test concentration (33,34).

The spectrum of the allergenic preservatives also varies from country to country. For example, in contrast to continental Europe where reactions to the methyl-(chloro)-isothiazolinone mixture and, more recently, methyldibromo glutaronitrile have been the most frequent (12,13,30,31,35), in the United Kingdom, formaldehyde and its releasers have always been much more important, particularly as concerns quaternium-15 (30), although its incidence seems to have slightly decreased of late (36). 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 (37); this is often the case for other ingredients also. For instance, a young lady, after having previously been sensitized to mefenesin in a rubefacient, presented with an acute contact

Table 2 Twenty-six Substances to be Labeled Regardless of Function and Origin

References in Annex III of the cosmetics directive	Name in the cosmetics directive	INCI name
(67)	Amyl cinnamal	Amyl cinnamal
(68)	Benzyl alcohol	Benzyl alcohol
(69)	Cinnamyl alcohol	Cinnamyl alcohol
(70)	Citral	Citral
(71)	Eugenol	Eugenol
(72)	Hydroxy-citronellal	Hydroxycitronellal
(73)	Isoeugenol	Isoeugenol
(74)	Amyl cinnamyl alcohol	Amylcinnamyl alcohol
(75)	Benzyl salicylate	Benzyl salicylate
(76)	Cinnamal	Cinnamal
(77)	Coumarin	Coumarin
(78)	Geraniol	Geraniol
(79)	Hydroxymethylpentyl-cyclohexenecarboxaldehyde	Hydroxyisohexyl 3-cyclohexene Carboxaldehyde
(80)	Anisyl alcohol	Anise alcohol
(81)	Benzyl cinnamate	Benzyl cinnamate
(82)	Farnesol	Farnesol
(83)	2-(4-tert-butylbenzyl) propionaldehyde	Butylphenyl methylpropional
(84)	Linalool	Linalool
(85)	Benzyl benzoate	Benzyl benzoate
(85)	Hexyl cinnamaldehyde	Hexyl cinnamal
(86)	Citronellol	Citronellol
(88)	d-Limonene	Limonene
(89)	Methyl heptin carbonate	Methyl 2-octynoate
(90)	3-Metyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	α-isomethyl ionone
(91)	Oak moss extract	EU: *Evernia prunastri* U.S.A.: *Evernia prunastri* (oak moss) extract
(92)	Tree moss extract	EU: *Evernia furfuracea* U.S.A.: *Evernia furfuracea* (tree moss) extract

dermatitis on the face at the first application of a new cosmetic cream containing chlorphenesin, which was used as a preservative agent (data on file). Apparently, it is a potential sensitizing agent (38) and 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 Europe (30). Sodium metabisulfite, present in oxidative hair dyes (data on file), may cause allergic contact dermatitis both to the clients and to the hairdressers.

"Active" or Category-Specific Ingredients

With regard to "active" or category-specific ingredients, in contrast to de Groot (3), we found an increase in the number of reactions to oxidative hair dyes (paraphenylenediamine or PPD and related compounds) during the period 1991 to 1996, as 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 a PPD base—a more appropriate screening agent for PPD allergy—may also have influenced the incidence (39). They are important causes of professional dermatitis in hairdressers who also often react to allergens in bleaches (persulfates, also causes of contact urticaria) and permanent-wave solutions (primarily glyceryl monothioglycolate, which may provoke cross-sensitivity to ammonium thioglycolate) (40,41). 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 (42). Acrylates (methacrylates) are also causes of reactions to artificial nail preparations, more recently to gel formulations, with both manicurists and their clients (43).

Moreover, "natural" ingredients may induce contact allergic reactions as well. Some examples are butcher broom (*Ruscus aculateus*), which is also a potential allergen in topical pharmaceutical products (44), hydrocotyl (asiaticoside) (45), and panthenol (46). 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 (47).

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 (3,30,48–50). Indeed, isopropyl dibenzoylmethane was even withdrawn for this reason (3). 4-Methylbenzylidene camphor, cinnamates, and phenylbenzimidazole sulfonic acid are only occasional, sometimes even rare, causes of cosmetic reactions. The use of para-aminobenzoic acid and its derivatives has decreased considerably. Contact allergic reactions to them were generally related to their chemical relationship to para-amino compounds (51), although they were also important photosensitizers (48).

In our experience (12,13,30), the contribution of sunscreens to cosmetic allergy is relatively small, despite the increase in their use as a result of the media attention 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 photo-allergy 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 (52), 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). 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 cocamidopropyl betaine, an amphoteric tenside mainly present in hair- and skin-cleansing products. Whether the compound itself or cocamidopropyl dimethylamine, an amidoamine, or dimethylaminopropylamine (both intermediates from the synthesis) are the actual sensitizers, is still a matter of discussion (53,54). It is also not clear whether cocamidopropyl-PG-dimonium chloride phosphate (phospholipid FTC (55), an allergen in skin care products, can cross-react with cocamidopropyl betaine. Other emulsifiers and vehicle components that were more recently found to be contact allergens in cosmetics are maleated soybean oil (56), butylene glycol and pentylene glycol (aliphatic alcohols with similar uses to propylene glycol that is considered to have more irritant and allergenic effects) (57,58), ethylhexylglycerin (syn.: octoxyglycerin) (59), methoxy PEG-17 and PEG-22/dodecyl glycol copolymers (alkoxylated alcohols and synthetic polymers) (60), and alkylglucosides (condensation products of fatty alcohols with glucose) (61,62).

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 (63).

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 with all its ingredients. We can only find the allergens we look for. There are several guidelines for skin tests with cosmetic products that the patients

supply themselves (64). Not only the patch and photo-patch tests, but also semi-open tests, usage tests, or repeated open application tests may be needed to be performed to obtain a correct diagnosis.

HYPOALLERGENIC PRODUCTS

Most of the cosmetic industry is making a great effort to commercialize safest possible products. Some manufacturers market cosmetics containing raw materials that have a "low" sensitization index or a high degree of purity, or from which certain components have been eliminated (5,65) (generally perfume ingredients). Sometimes active preservative agents are also omitted, and in sunscreens immunologically inert physical agents are being used more often than chemical ultraviolet absorbers.

Statements such as "recommended by dermatologists," "allergy-tested," or "hypoallergenic" have been put on the packaging material 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 (66).

The latest trend is target marketing to people with a "hypersensitive" or "intolerant" skin, a term often used for the shadowy zone between normal and pathological skin. These would be the 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, those suffering from seborrheic dermatitis (67), 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 (68,69), 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 "quasi-drugs."

The meaning of most such claims used nowadays is unclear, both for the dermatologist (65–67) and the consumer, the latter being convinced that the hypersensitive skin is the 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, at least the contact allergic types, because people sensitive to specific ingredients must avoid products containing them.

Therefore, ingredient labeling can be of tremendous help. Providing the allergic patient with a limited list of cosmetics that can be used is practical and effective (70).

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 the adverse reactions to cosmetic products is critical in a product design. Apparently, premarketing studies are unable to identify all the pitfalls. Therefore, 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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54 Operational Definition of a Causative Contact Allergen—A Study with Six Fragrance Allergens

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INTRODUCTION

Contact allergic dermatitis remains a significant public health problem. Its diagnosis and prevention is complicated by the difficulty of identifying allergens responsible for a patient's condition (i.e., those that have actually caused the allergic contact dermatitis). This paper attempts to provide criteria that can be used as an operational definition of causative allergens. Six fragrance allergens exemplify the application of these criteria. Each is discussed in a separate paper (1–6).

Although predictive tests can identify potential allergens, it is only through clinical diagnosis studies on patients with current contact dermatitis that truly causative allergens can be identified. This is, however, not a simple matter and is complicated by practical difficulties inherent to the technique of patch testing, to the physiological nature of type IV allergy, and by other practical matters such as the available time and willingness of patients to submit to prolonged studies.

Clinical patch testing remains a partially subjective field (7). When a clear reaction is observed, it is not always certain that it has been due to a truly clinically –relevant allergic response. Marks et al. suggested that more than 40% of 3000 patients with suspected allergic contact dermatitis were, in fact, suffering from irritant or some other form of nonallergic contact dermatitis (8). A recent study has shown that there was an association between erythematous reactions to some allergens and irritant reactions to sodium lauryl sulfate (SLS), which is a putative marker for hyperreactive skin, thus allowing many reactions of this type to be classified as irritant rather than allergic in nature (9).

Apart from false-positive reactions from irritancy, there is always a possibility that other false-positive reactions can occur from cross-reactions where patients react to substances, which are not the primary sensitizers and which initially induced the allergic state (10). Similarities in chemical structure or cutaneous metabolism would appear to be major factors in cross-reactivity (11). Over 70 different cross-reacting pairs or families of fragrance ingredients have been catalogued (12,13). Positive correlations of concomitant reactions in different pairs of components of the fragrance mix have also been recorded in poly-reactive patients (14).

False-positive reactions can also arise from phenomena such as the "excited skin syndrome" that occurs after a number of patches result in positive test results, which cannot be reproduced when the patient is retested (15,16). Over 40% of such positive patch reactions are lost on repatching (16–18). Indeed, some studies have involved phased patch testing schedules to avoid false positives due to this syndrome.

Even when there is clear evidence that the reaction is allergic in nature, ascertaining the clinical relevance of the patch test requires knowledge of technical aspects relating to specificity and sensitivity issues (19). In one of the more familiar clinical correlations—nickel allergic contact dermatitis—there is a high ratio of false-positive and false-negative reactions (20). Even when an allergic reaction has been indicated, the chances that an experienced physician will accurately identify the causative allergen from clinical information is about 50% mostly when common allergens are involved, but this is reduced to 10% for less common allergens (21).

There is increasing evidence that diagnostic patch testing may also elicit true allergies, but these allergies are not the cause of the patient's current contact dermatitis. Lachapelle has defined clinical relevance as "the capability of an information retrieval system to select and retrieve data appropriate to a patient's need" (22). In this context, Lachapelle has distinguished

between past (not directly related to the patient's current problems) and current relevance and has devised a system for distinguishing between the two. He has also defined the need to determine the "intrinsic imputability" of a suspected allergen as the "possible (and not necessarily exclusive) cause-effect relationship between each positive test to an allergen and the occurrence of a given chemical event." The approach proposed here uses some aspects of this system. A more recent publication (23) gives strategies for determining true clinical relevance. These require establishing the existence of past exposure and ensuring that the patient's exposure has indeed been responsible for the observed dermatitis. A number of suggestions are also made for improving evidence-based diagnosis of relevance. These include running use tests with implicated products, accounting for possible cross-reactions and a more rigorous and detailed examination of the case in which the clinician must retrieve the pertinent historical data, trace the responsible environmental exposure, and perform the appropriate tests.

While some allergies revealed by patch testing may pertain to past allergic clinical events, others may pertain to allergies acquired by the patient but which have never been clinically manifested (i.e., have never caused contact dermatitis in the patient) (24). There is increasing experimental evidence, mainly in animal tests (25–30) but also in some older studies on humans (31) to show that the threshold dose of elicitation varies in accordance with the conditions of induction. When induction conditions are severe, the elicitation threshold dose is low. When induction occurs under mild conditions, much higher exposures are required to elicit an allergic reaction. This means that it may be possible for patients to have acquired allergies under low exposure conditions (e.g., use of cosmetics or other consumer products resulting in relatively mild exposures to their allergenic components), which will never be elicited during their everyday lives as long as exposure remains low. However, these allergies may be artificially elicited under higher exposure conditions experienced in patch testing. These true positive reactions may not be clinically relevant and indeed may not represent a cause for concern for the patient as they reflect an allergic state, which may never manifest itself clinically.

Diagnostic patch tests are by necessity, purposefully designed to avoid false negatives (i.e., to avoid missing possible causative agents), and to do this, the patch test conditions are intentionally more severe than normal exposure conditions. We have clarified that the patch test dose (single application) is usually higher than use test dose (32,33). This can be seen by comparing relative doses. Taking fragrance allergens as examples, a fragrance ingredient used at 1% in a perfume spray (the product type that produces the highest on-skin level of fragrance), a maximum dermal loading of 26 $\mu g/cm^2$ is obtained (34).[a] Yet the use of diagnostic patch tests with 1% of the same ingredient in 19 mm Hill Top Chambers® will deliver a skin loading of 1770 $\mu g/cm^2$ (35), a 68-fold increase. The use of 2 × 2 cm Webril® patches, 8 mm Finn Chambers®, and a Professional Products® 1.9 × 1.9 cm patch would result in 38-fold, 11-fold, and 21-fold increases, respectively. To this, we should add the (dose enhancing) potentiating effects of occlusion [numerous publications (19,36–39) and the potentiation due to an exceptionally long duration of the 48-hour exposure (39)]. The intentions behind this type of exaggeration are laudable, being aimed at identifying substances to which the patient may be allergic. However, they will not necessarily identify the substance that is primarily responsible for the allergy from which the patient is suffering at that specific time. We return to this particular point in the section "Were These Sufficiently Maximized".

Defining the appropriate concentrations for patch tests balances

(i) nonirritation;
(ii) avoiding active sensitization, and
(iii) an appropriate enhancement of concentration that will identify an allergic individual.

The situation with fragrance allergens remains complex because of the relatively limited data on which to define appropriate patch test concentrations, and until recently, the relative difficulty of obtaining documentation that a given consumer product contains the individual fragrance ingredient and its concentration. However, the International Fragrance Association

[a]This would correspond to a fragrance ingredient present at 20% in a fragrance used at 5% in this type of product.

(40) has recently carried out a number of industry-wide surveys, which have been aimed at determining the highest concentrations currently used in fine fragrance products (i.e., the type of consumer products, which delivers the highest levels of fragrance in terms of concentration and quantity per unit area). On the basis of these levels, we propose in the table (Annex) concentrations that could be used as guides to determine the concentrations in some common patch test systems. These would correspond approximately to the maximum exposure that could be expected from using a consumer product when the dermatologist is confronted with a case of allergic contact dermatitis but has no culprit product to examine.

Depending on whether a response is irritant or allergic has long been a complex challenge. All too frequently, inadequate (nonallergic) controls are available. Excited skin syndrome (15) provides further complexity; most clinicians do little single patch tests to verify this possibility. Brasch (41) and Geier (9) have suggested using their reaction index/positives ratio as a retrospective aid in defining those allergens whose positive responses might, in fact, be irritant instead.

In the ideal, but rarely encountered, clinical situation, the causative role of an allergen will be suggested by its presence in a consumer product that has already been identified as the cause of the patient's allergy. In such cases, the patch test should be conducted at a concentration that is related to the concentration of the suspected allergen in the product.

We suggest below the criteria by which the causative role of an allergen can be attributed to a specific case of clinical contact dermatitis. By far the more important is diagnostic patch testing as this alone can link the substance to the case. The criteria are aimed at determining the degree of confidence we can ascribe through diagnostic patch testing, a specific allergen's causal role in a specific case of allergic contact dermatitis. It is also important, however, to have some measure of the substance's intrinsic allergenic potency. For this reason, we are also presenting a scheme for ascribing a degree of confidence to the results of predictive tests.

These schemes build on a previous approach of Benezra et al. (10), and in subsequent papers; these criteria are put into practice by taking six fragrance allergens recently identified as major fragrance allergens (42). These have been specifically chosen to span the range of likely causality shown by the 24 allergenic substances identified in this new legislation. Two of the substances are considered as frequently encountered allergens (geraniol and amylcinnamic aldehyde), two are among the less frequently encountered (α-iso-methylionone and anisyl alcohol), and two are intermediate in this regard (citronellol and linalool).

DATA OBTAINED FROM DIAGNOSTIC PATCH TESTING OF PATIENTS IN DERMATOLOGICAL CLINICS

The following scoring system is proposed.

Is It A Primary Case Report or A General Review?

Many publications in this area are reviews or statistical studies of patch test results already published elsewhere. While these papers perform an important function, they present a possible source of duplicate reporting whereby the same patch test result is referred twice in the literature. It is therefore important to distinguish between these two types of studies. A higher degree of confidence is attributed to detailed primary studies.

Is Information Provided on the Number and Condition of the Patients?

The number of patients being examined is of importance mainly for epidemiological studies. However when studies do not provide information on the number and nature of the test materials to which individual patients reacted, then it is impossible to estimate the degree of poly-reactivity and possible cross-reactions. It is also important to know if the patients suffer from current eczema or other diseases. A lower degree of confidence is attributed when this information is not provided.

Are the Conditions of Patch Testing Given?

This is of primary importance particularly with regard to the purity of the test material (43). The test material should be clearly identified and degree of purity specified. The presence of potentially more allergenic impurities (e.g., aldehydes in alcohols and autoxidation products)

should also be controlled and indicated. A lower degree of confidence is attributed to results from studies where such verifications are not reported.

Ideally, the report should give a detailed description of the patch test conditions. The type and size of the patch, duration of occlusion, nature of the vehicle, and the concentration of the test material are a minimum of information that is required. The bioavialability of the test material and the dose in quantity per unit area will vary from one type patch test kit to another (35,44). The timing of readings is also of importance.

Unless it is clear from historical evidence, it is necessary to rule out the possibility that irritant reactions are observed. For this reason, it has been recommended to undertake a preliminary test of at least three concentrations on control subjects (45).

A higher degree of confidence is also attributed when it is clear that the patch test conditions have exposed the patient to levels of the allergen that are not disproportionately high with regard to the levels of exposure, which were suspected to have led to the patient's condition. Although there is no clear test to determine whether a positive patch test reaction has revealed the cause of the allergy or some latent subclinical allergy, information on the conditions of patch testing provides valuable information in making a judgment with any degree of confidence in this regard.

Are the Results Reported in Sufficient Detail?

The intensity of positive reactions should be recorded. Numerous authors have expressed concern that a significant proportion of patch test reactions may be irritant in nature. This is particularly the case with weakly positive (1+) scores (46), and it has been proposed that these scores should be handled separately (47). Studies comparing True Test TM and Finn Chambers showed that the fragrance mix gave about 47% irritant and questionable reactions with the former and about 45% with the latter, with a high degree of discordance between the two systems. In other large studies (48,49), about 60% to 70% of the reactions recorded were 1+, and it was speculated that up to 40% were irritant in nature (48). Further, the fact that the skin is not viewed for 24 hours following application of the patch makes it almost impossible to distinguish between quick-developing irritation and delayed contact hypersensitivity.

Scores should be given in a way that allows comparison of reactions experienced by a given patient to different test materials. A higher degree of confidence is attributed to results from studies where this information is provided.

Is It Possible to Ascertain if Patients had Reacted to Other Materials?

Concomitant reactions cloud the issue of causality. Although it is possible that a particular case of allergic contact dermatitis has been caused by several allergens, other explanations for multiple reactions to different suspect allergens will need to be ruled out. For this reason, it is important to know the other substances, which produced positive patch test reactions in each patient.

Can We Rule Out Cross-Reactions?

A lower degree of confidence is attributed to results from studies where uncertainty arises in this regard. When positive reactions occur to different substances in the same patient, and these substances have similar chemical structures, the possibility arises that some of these patch test reactions are in fact false indicators of the true cause of the case of contact allergic dermatitis.

Can We Rule Out "Excited Skin"?

There is also the possibility that false-positive reactions appear because of the excited skin syndrome (15–19). The likelihood of this occurrence can be reduced by carrying out patch tests in a time-phased manner so that the number of patches is minimized. A higher degree of confidence is allocated to studies that have taken such precautions or other measures to ensure that some of the observed reactions are not due to artifacts of this type.

Has the Substance Been Tested in Usage Tests?

Although some uncertainties in these techniques need to be resolved (50), the use of repeat open applied tests (ROATs) and provocative use tests (PUTs) can add important extra evidence of the causative role as they confirm under milder conditions than patch testing the

allergenic role of the substance. If these can be performed on suspected sources of exposure to the patient (e.g., consumer products containing the substance), an even higher degree of confidence is attained (see following section). This is a key to the scheme proposed by Lachapelle (22) for assigning intrinsic imputability.

Has the Substance and the Allergy Been Linked to a Specific Consumer Product or Exposure Situation?

This is perhaps the gold standard for establishing the causal culpability of a suspect. Ideally, this would go further than simply gathering information of the possible causative products [the third criteria of Lachapelle (22)] and include carrying out patch tests on fractions of the(se) causative product(s) until the culpable allergens have been identified by producing reactions to the exclusion of all other components. A number of studies producing convincing results of this type have been published [e.g., Handley and Burrows (51)]. However in such cases, the highest degree of confidence can only be ascribed when it is clear that the patch test conditions have exposed the patient to levels of the allergen, which are not disproportionately high with regard to the levels of exposure, which were suspected to have led to the patient's condition.

The following scoring system has been used.

5: meets all criteria
4: meets all criteria but number of cases is marginal
3: meets criteria but some parameters questionable
2: evidence does not unambiguously indicate causative role of the test substance
1: fails several criteria, results are not considered to be reliable
0: fails all criteria/not primary report in literature of cases cited

DATA OBTAINED FROM PREDICTIVE TESTS IN ANIMALS AND HUMAN VOLUNTEERS

The first part of this analysis examines the likelihood that the designated substance shows an inherent potential to sensitize. This type of information is best obtained from predictive tests, although it should be cautioned that these data only provide information on a substance's intrinsic hazard. The conditions of exposure to the general public may be sufficiently high in some instances to produce reactions in substances, which fail to show any significant sensitization hazard (52). For this reason, the apparent absence of a sensitization potential from predictive tests will not rule out the likelihood that a substance will not cause reactions in sufficiently exposed populations. Given this proviso, we suggest that the degree of confidence should be attributed to predictive tests according to the following criteria.

Was the Test Material Clearly Identified?

The test material should be clearly identified, and the degree of purity should be specified. The presence of potentially more allergenic impurities (e.g., aldehydic impurities in alcohols and autoxidation products) should also be controlled. A lower degree of confidence is attributed to results from studies where such verifications are not reported.

The Type of Test/Type of Test Subjects

The type of test and nature of the test subjects should be clearly specified. If these are human subjects, their dermatological status should have been verified by a dermatologist prior to and, if necessary, during the course of the study. It is also accepted that some types of test are more sensitive than others. Adjuvant tests in guinea pigs have long been regarded as more sensitive than non-adjuvant methods. Some tests have been less well validated than others. A lower degree of confidence is attributed to results from studies that are not as sensitive as others.

Were Details of Test Conditions Provided?

Information on the exact protocols should be provided to ensure that the most sensitive methodology has been applied. The test concentration (and the skin loading as expressed in quantity per unit area) should be neat to the limit of irritation. The choice of the vehicle(s) and type of patches can also have an influence on the sensitivity of the test. A lower degree of confidence is attributed to results from studies where this information is not reported or

deemed less than optimal. Officially approved test protocols should be used for those methods that have been codified in this way [e.g., by Organisation for Economic Co-operation and Development (OECD), 1981 as amended (53)]. There is also accumulating evidence to show that occlusion and ethanol may in fact produce false positives in human studies. This should also be taken into account.

Were These Sufficiently Maximized?

A lower degree of confidence is attributed to results from studies that are deemed to be less than optimally maximized to avoid false-negative results.

Were There Adequate Controls?

These are necessary to ensure that irritancy is not occurring during induction. Tests involving a challenge phase should include challenges to naive subjects to control irritancy. Where ethically possible, the laboratory performing these studies should carry out regular positive control studies using standard borderline allergens. A lower degree of confidence is attributed to results from studies where these controls are not reported to have been used or where they have also produced reactions.

Was the Number of Test Subjects Sufficient?

There are international standards requiring the minimum number of animals to be used in some tests [OECD, 1981 as amended (53)]. Tests on human subjects should generally involve more than these because of the inherent and environmental variability of the test subjects (54), and ideally at least 200 should be used. A lower degree of confidence is attributed to results from studies in which an insufficient number of test subjects were used.

Were the Results Presented in Sufficient Detail?

The intensity of positive reactions should be recorded. Scores should also be followed for individual test subjects to ensure that, for instance, those reacting at one challenge are the same as those who react at subsequent challenges. A lower degree of confidence is attributed to results from studies where this information is not provided.

SCORING

The following scoring system is used:

5: meets all criteria
4: meets all criteria but number of positives is marginal
3: meets criteria but some parameters questionable (e.g., insufficient data provided or test not fully maximized)
2: controls apparently absent or small number of test subjects
1: fails several criteria, results are not considered to be reliable
0: fails all criteria

CONCLUSIONS OF THE STUDIES ON SIX FRAGRANCE ALLERGENS

The accompanying papers on amylcinnamic aldehyde, anisyl alcohol, citronellol, geraniol, linalool, and α-iso-methylionone (1–6) show that when the underlying clinical and experimental data are analyzed according to the criteria outlined above, a clear cause-effect relationship has infrequently or rarely been established and would not necessarily be expected on the basis of the generally weak sensitizing potential of these substances coupled with reasonably low exposure conditions. This is not to say that some of these substances are frequent inducers of type IV allergy in members of the public. It remains to be seen however, how often such allergy, once established, is responsible for any of the cases of allergic contact dermatitis commonly ascribed to these substances.

Schnuch and others have commented extensively on the wisdom and criteria for definition of what is a chemical allergen in man. References (55–57) provide a state of the science.

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ANNEX: PATCH TEST CONCENTRATIONS THAT CORRESPOND TO THOSE EXPERIENCED IN MAXIMUM CONSUMER EXPOSURE

	Maximum exposure from cosmetics		Equivalent concentrations in standard patch test kits[a]			
Test material	Concentration[b]	Quantity/ unit area[c]	Finn Chambers® (8 mm)	Hill Top Chambers® (19 mm)	Professional Products® (1.9 × 1.9 cm)	Webril® (2 × 2 cm)
Amylcinnamique aldehyde	0.89%	23 μg/cm^2	0.08%	0.013%	0.04%	0.023%
Anisyl alcohol	0.57%	15 μg/cm^2	0.05%	0.008%	0.03%	0.015%
Citronellol	0.70%	18 μg/cm^2	0.06%	0.01%	0.03%	0.018%
Geraniol	0.62%	16 μg/cm^2	0.05%	0.009%	0.03%	0.06%
Linalool	0.86%	22 μg/cm^2	0.075%	0.013%	0.04%	0.022%
α-iso-methylionone	0.74%	19 μg/cm^2	0.064%	0.011%	0.035%	0.019%

[a]Does not take account of additional effects of occlusion and 48-hour duration of patch tests. Based on data from Robinson et al. (34)
[b]Data used by RIFM from IFRA surveys. Assumes 20% of fragrance in the cosmetic (fine fragrance product).
[c]From Gerberick et al. (33). Spray-on fragrance product delivers a maximum of 2.6 mg product/cm^2.
. Finn Chambers® (8 mm): 30 mg/cm^2, Hill Top Chambers® (19 mm) 177 mg/cm^2, Professional Products® Patch (1.9 × 1.9 cm): 55.4 mg/cm^2, Webril Patch® (2 × 2 cm): 100 mg/cm^2.
Abbreviation: IFRA, international fragrance association.

55 Anti-Itch Testing: Antipruritics

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INTRODUCTION

Itching, or pruritus, is an unpleasant sensation that provokes a desire to scratch. Chemical, mechanical, thermal, and electrical stimuli can elicit itch (1–5). Mediators of itch, presumably, directly act on nerve fibers or lead to a nerve stimulation cascade whose final common pathway is interpreted in the central nervous system as itching (2–6). Putative receptors for itching are C-fibers with exceptionally low conduction velocities and insensitivity to mechanical stimuli (4–6). Histamine, the prototypical chemical mediator of itch, which is released during mast cell degranulation and mediates its effects in the skin via H_1 receptor (3,5), is the best-known experimental pruritogen (2,3,5,7). Other pruritogens such as compound 48/80 (8,9), substance P (10,11), and serotonin (12) have been studied.

Antipruritics may alleviate or diminish itching sensation. Topical antipruritics such as antihistamines, anesthetics, capsaicin, corticosteroids, and cooling agents are extensively used (8–10). To define antipruritic effects, testing methodologies have been developed (11–13). However, the clinical effects of anti-itch vary, and sometimes it is difficult to compare efficacy between antipruritics. One reason may be inadequate biometrics, as itch is a subjective symptom and to measure its severity is a challenge; its magnitude (intensity) can be only estimated from reports of patients or volunteers. Methodologies have been adopted to evaluate antipruritics that may aid future development of anti-itch products.

This chapter focuses on the evaluation of topical antipruritics; and, further reviews recent investigations involving thermal stimuli-modifying itch (14–16), electronic devices for measurement (8,9,17) as well as alleviation of itch (18), newly found use of known drugs (11,19), questionnaires for assessment of pruritus in atopic and uremic patients (20,21), and possible models for developing new antipruritics (10,11,17).

METHODOLOGIES

Histamine-Induced Itch Human Model

Rhoades et al. (22) examined the inhibition of histamine-induced pruritus by three antihistaminic drugs using a double-blind crossover study on 28 human subjects. These included: diphenhydramine HCl, cyproheptadine, hydroxyzine HCl, and a lactose placebo in identical capsules. All subjects were given intradermal injections of increasing doses of aqueous histamine phosphate in the volar aspect of the forearm to establish their individual threshold levels at which itching occurred. Following the establishment of a baseline, the subjects received two doses of one of the three antihistamines or placebo on four test periods with a one-week interval between test days. Results revealed a fivefold increase above baseline of the histamine dose required to produce pruritus following both cyproheptadine and placebo. This compared to a 10-fold increase following diphenhydramine and a 750-fold increase following hydroxyzine HCl.

Yosipovitch et al. (14,15,23–26) performed human studies to evaluate the antipruritics with this histamine injection, as well as histamine-iontophoresis-induced itch models in man. They also utilized the visual analog scale (VAS) to measure the itch magnitude (intensity). One study compared the effect of antipruritics of a high-potency corticosteroid, clobetasol propionate (CP) ointment versus its placebo in a double-blind manner on 16 healthy volunteers. Additionally, they evaluated the affect of CP and its placebo to thermal sensation and pain (23). They demonstrated that the CP had rapidly decreased histamine-induced itch, but did not alter warmth sensation and thermal pain thresholds. Another study determined

the effect of menthol and its vehicle (alcohol) on thermal sensations, pain, and histamine-induced experimental itch with 18 human subjects (24). Menthol showed a subjective cooling effect lasting up to 70 minutes in 12 of 18 subjects; however, it did not affect the cold and heat threshold, nor did it affect cold and heat pain threshold. Alcohol produced an immediate cold sensation lasting up to 5 minutes in 4 of 18 subjects and lowered the sensitivity of cold sensation threshold ($p < 0.05$). Histamine injection did not change thermal and pain thresholds. Menthol did not alleviate histamine-induced itch magnitude, or its duration. They suggested that menthol fulfills the definition of a counterirritant, but does not affect histamine-induced itch, nor does it affect pain sensation.

Later, they examined the effect of topical aspirin and its model vehicle dichloromethane on histamine-induced itch in 16 human subjects (25). Aspirin significantly reduced itch duration ($p = 0.001$) and decreased itch magnitude ($p < 0.04$). Aspirin and vehicle application did not affect thermal and pain thresholds during histamine-induced itch. Further, they tested the antipruritics effect and thermal sensation of a local anesthetic, 1% pramoxine, and its vehicle control in 15 human subjects (26); pramoxine significantly reduced both the magnitude and duration of histamine-induced itch. The pramoxine also reduced the cold pain threshold but did not affect warm sensation or heat pain threshold.

Recently, they investigated the effect of thermal modulation in histamine-induced itch (14,15). They first investigated the effect of thermal stimuli and distal scratching on skin blood flow and histamine-induced itch in 21 healthy volunteers (14). Thermal stimuli included 41°C, 15°C, and 49°C while scratching was performed using a 7-inch cytology brush. Assessment of itch was done psychophysically using computerized visual analog scale (COVAS, Medoc, Ramat Ishai, Israel), and mapping of skin blood flow was done utilizing a PIM II laser Doppler perfusion imager (LDPI) at baseline, in the different thermal stimuli, after histamine iontophoresis treatment, and after scratching. They found that scratching significantly ($p = 0.01$) reduced skin blood flow and itch; noxious heat significantly increased basal skin ($p \leq 0.001$), but was not significant in reducing blood flow and itch intensity; noxious cold and cooling significantly ($p = 0.007$) reduced itch intensity but not in histamine-induced skin blood flow; and subnoxious warming neither had an affect with both itch intensity nor skin blood flow. They suggest that heat pain and scratching may inhibit itch through a neurogenic mechanism that also affects blood flow.

The other study involved 21 healthy human volunteers and assessed whether (*i*) the sensory perception of itch is attenuated by interactions between thermal and mechanical stimuli, as well as afferent information related to itch; and (*ii*) if interindividual differences in itch perception were related to interindividual differences in pain sensitivity (15). They used a 100-mm COVAS on histamine-iontophoresis applied on the flexor forearms. After 30 seconds, thermal stimuli [noxious cold (2°C), innocuous cool (15°C), innocuous warmth (41°C), noxious heat (49°C)] were delivered repetitively in a random order by a 16 × 16 mm Peltier device at 3 cm distal to the sight of histamine-iontophoresis. Cytology brush was used to simulate scratching at a constant pressure. Results revealed that noxious heat, noxious cold, and scratching significantly ($p < 0.004$, $p < 0.001$, $p < 0.0001$, respectively) reduced itch via spinal or supraspinal mechanism. A possible explanation favoring supraspinal mechanism is that these three stimuli were sufficient to have called attention from the prefrontal cortex, thus diverting attention away from the itch. The study revealed significant interindividual differences in itch sensitivity to histamine. On the other hand, interindividual differences in itch sensitivity were unrelated to interindividual differences pain sensitivity.

Pfab et al. also used thermal modulation for histamine-induced itch (16). They evaluated the effect of short-term alternating temperature modulation in nine healthy human volunteers and developed a possible methodology for imaging studies using functional magnetic resonance imaging. Histamine induction was done using the skin prick model (27) to the volar aspect of the dominant right forearm of each subject. Skin temperature was modulated, intensity of itch was determined, and the Eppendorf Itch Questionnaire was done by all subjects at the end of the study. Results revealed the mean itch intensity was significantly ($p < 0.001$) higher in the 25°C temperature compared with that in 35°C temperature. Alternating changes in mean itch perception between 25°C and 35°C were notably reproducible. And, the mean descriptive and emotional ratings were also significantly ($p < 0.01$) higher in the 25°C temperature compared with that in 35°C temperature as well. They concluded that a decrease in short-term moderate temperature enhances histamine-induced itch, providing the possibility of further and more

detailed itch investigation by methods usually used for nociception such as functional magnetic resonance imaging.

Weisshaar et al. (28) evaluated the effect of topical capsaicin on the cutaneous reactions and itching to histamine in atopic eczema (AE) patients and healthy human subjects. Capsaicin 0.05% was applied three times daily over a five-day period to the same infrascapular region. The effects of pretreatment upon the pruritogenic and wheal and flare reactions to subsequent histamine iontophoresis were evaluated on the following day. In control subjects, but not in AE patients, capsaicin pretreatment significantly reduced the flare area. Compared with control subjects, AE patients showed a lack of alloknesis (itchy skin) or significantly smaller areas of alloknesis in pretreated and nonpretreated skin. In control subjects, capsaicin pretreatment significantly reduced itch sensations compared with nonpretreated skin, whereas in AE patients no differences were seen. Itch sensations in capsaicin-pretreated skin were significantly lower in control subjects than in AE patients. They concluded that capsaicin effectively suppresses histamine-induced itching in healthy skin but has less effect in AE. The diminished itch sensations and the absence of alloknesis in atopic individuals indicate that histamine is not the key factor in itching in AE.

Thomsen et al. (29) conducted a randomized, double-blind, and placebo-controlled human study to determine the antipruritic ability of topical aspirin in inflamed skin. In 24 nonatopic volunteers, an inflammatory skin reaction was induced in forearm skin at five sites by sodium lauryl sulfate (SLS) contained in Finn Chambers. Aspirin 10%, aspirin 1%, mepyramine 5%, and vehicle were applied to the inflamed and corresponding noninflamed areas 20 minutes before itch induction with intradermal histamine injection. No difference in itch intensities was found after application of aspirin, mepyramine and vehicle, but more itch was induced in aspirin and mepyramine pretreated sites in inflamed skin compared with normal skin ($p < 0.05$). In normal skin, flare areas were smaller after pretreatment with aspirin 10% ($p < 0.05$) and mepyramine ($p < 0.001$), as were wheal areas after mepyramine ($p < 0.01$), compared with vehicle pretreatments. In inflamed skin, flare areas were smaller after pretreatment with aspirin 10% ($p < 0.01$) and mepyramine ($p < 0.001$), as were wheal areas after aspirin 10% ($p < 0.01$), aspirin 1% ($p < 0.05$), and mepyramine ($p < 0.001$). They concluded that despite a significant skin penetration as measured by the influence on wheal and flare reactions, topically applied aspirin did not decrease histamine-induced itch in the model used.

Zhai et al. (30) evaluated the antipruritic effect of hydrocortisone (1% and 2.5%) and its vehicle control on histamine-induced itch and sensory effects in 18 human subjects. In comparison with placebo, 2.5% hydrocortisone significantly ($p = 0.03$) reduced itch duration from 12.6 ± 11.0 to 8.6 ± 8.2 minutes (the reducing rate was 32%) as well as itch magnitude (at minutes 3, 6, 7, and overall). Placebo, 1% and 2.5% hydrocortisone significantly altered ($p < 0.05$) the cold sensation threshold. No treatment altered cold or heat pain thresholds. They suggested that topical application of 2.5% hydrocortisone might be significantly beneficial for the treatment of histamine-induced itch.

They further ascertained the antipruritic effects of topical strontium salts with the histamine-induced-itch model on eight human subjects (31). Strontium nitrate, in comparison with its vehicle control, significantly shortened itch duration from 28.1 ± 5.4 to 18.5 ± 4.2 minutes ($p < 0.01$) and reduced itch magnitude at time points 12 to 20 minutes and overall ($p < 0.05$). They concluded that strontium nitrate may act as a topical antipruritic agent in reducing histamine-mediated itch. Furthermore, they utilized this histamine-induced itch human model to screen and to compare the efficacy of a group of topical antipruritics on 10 individuals who were responsive to histamine-induced itch sensation (32). The pramoxine-containing cream (formulation D) significantly ($p < 0.05$) decreased itch magnitude (within a 20-minute test period), from 2.6 ± 2.1 to 2.2 ± 2.1 cm when compared with its vehicle control; it also significantly ($p < 0.05$) shortened itch duration (15.0 ± 7.4 minutes) in comparison with its vehicle control (20.3 ± 7.0 minutes). Of all the formulations tested, pramoxine-containing cream was the most effective antipruritic in decreasing histamine-induced itch.

Kesting et al. established a reliable model that objectively demonstrates the effectiveness of ear electro-acupuncture by reducing alloknesis areas in experimentally induced itch (18). Thirty-two human healthy volunteers underwent two experiments, both their volar forearms treated with histamine iontophoresis. In the first experiment, 16 were administered with electrical ear acupuncture on the left ear, and the other half, the right ear. Alloknesis were

measured at 5 and 10 minutes posthistamine iontophoresis treatments. In the second experiment, none of them received acupuncture, serving as control. Results revealed after the 5th and 10th minute, the alloknesis areas in the ipsilateral sites, treated with acupuncture, were significantly smaller ($p < 0.05$) than the contralateral untreated sites. And, the contralateral sites of the first experiment compared with the results of the second experiment showed increasing areas of alloknesis in both the 5th and 10th minute, and were not statistically significant in terms of the size of the increasing alloknesis areas.

Scratch Behavior Measurement

Tohda et al. (33) studied the effect of Byakko-ka-ninjin-to (BN), which is composed of gypsum, the root of anemarrhena, ginseng, licorice, and rice on the inhibition of itch using naive/challenged (NC) mouse model of atopic dermatitis (AD). BN (200 mg/kg, p.o.) significantly inhibited the scratching frequency in NC mice, and decreased the skin temperature by 1.97°C.

Electronic Devices for Accurate Measurement of Itch

Orito et al. developed a model for assessing the duration of scratch behavior in mice by evaluating the time course changes in the distance between the animal's hind limbs and the back of the neck. (8) Ten micrograms ($\sim$10 μL) intradermally administered compound 48/80 was used to induce itch to the backs of ICR mice, and their scratch behavior was recorded on digital videotape, as well as the distance between the back and the hind limb (hind limbs were color coded) was measured continuously using an image analysis system (SCLABA system, *Noveltec*, Kobe, Japan). Results for "true" scratching behavior revealed no significant difference among the three thresholds ($p = 0.1$); while the results for duration of scratching recorded during the observation period increased significantly ($p < 0.001$) as the threshold lengthened. This study suggests that the SCLABA system is a good tool for studying factors which may cause itch, and also for evaluation of efficacy of a new antipruritic drug using experimental animals such as NC/Nga mice, a representative model of AD.

Inagi et al. evaluated and characterized scratching behavior using their new apparatus, MicroAct in ICR and BALB/c mice (9). Inductions of scratching behavior were done by: (*i*) intradermally injecting 20 μL of compound 48/80 and 20 μL of physiologic saline in two sets of both ICR and BALB/c mice, the other set served as control; and (*ii*) intradermal administration of 20 μL of appropriately diluted anti-dinitrophenyl (anti-DNP) monoclonal IgE to induce passive cutaneous anaphylaxis (PCA); and (*iii*) to induce contact sensitivity reaction, nine applications of 0.15% of DNCB diluted in acetone were done on the backs of BALB/c mice. Frequency of scratching events ($\geq$3 consecutive scratch behavior or beats), total scratching time, and total number of beats (scratch behavior) detected by MicroAct were the parameters used. Results revealed that MicroAct's tally were comparable with the observer's tally. The frequency of scratching events and total scratching time increased in a dose-dependent manner for both the ICR and BALB/c mice. In the PCA of the ICR mice, the three parameters increased, though not significantly. There was a significant ($p < 0.001$) increase in the three parameters in the induced contact sensitivity in the BALB/c mice.

Benjamin et al. developed a practical method for evaluating scratch behavior by use of a portable digital limb-worn accelerometers suitable for children and adults, in seven atopic children (aged 2–9 years), and seven children (aged 5–7 years) without atopy, utilizing a night video-recording with infrared light as the gold standard (17). Parameters of measuring accelerometer readings were epoch (unit of time assayed) equivalent to two seconds and "burst analysis" (successions of 1 epoch); while for the night video recording were observed as sleeping, scratching, restless movements, and movements under covers–which were clearly defined operationally in the experiment. Results from night video recording revealed a statistically significant ($p < 0.01$) 46-minute less sleep and a greater "scatter" of readings in atopic patients compared with the control group; while results from the accelerometer readings were significantly ($p < 0.01$) clear and consistent, and, though not significant, arm movements resulted higher than lower limbs. Accelerometer scores were highly correlated with the video scores (< 0.01), for scratching, restlessness, and sleeping time.

Others

These include contact allergic dermatitis model (poison ivy), contact irritant dermatitis induced by SLS, etc. (34–36).

Newly Found Use of Known Drugs

Substance P-induced itch was used by Liebel et al. and found that sertaconazole nitrate inhibited contact hypersensitivity and scratching responses in a murine model of pruritus (11). Fifty microliter (50 μL) of 300 μg of substance P dissolved in sterile physiological saline was intradermally injected in male mice to produce itch response, while 50 μL intradermal administration of sterile physiologic saline served as control. Results revealed statistically significant reduction in scratching with sertaconazole nitrate-treated animals ($p < 0.05$), compared with the reduction in scratching in 1% hydrocortisone-treated animals.

Wikström et al. conducted a randomized, double-blind, placebo-controlled study, using κ-opioid agonist nalfurafine in 144 uremic patients with ESRD undergoing hemodialysis (19). Itch intensity was assessed using a five-point scale and revealed a significant ($p < 0.0410$) reduction in itch intensity, as well as the number of excoriations in the body using a three-point scale showed a significant ($p = 0.0060$) reduction. Safety profiles of nalfurafine were evaluated and showed the most common adverse drug reactions were headache, insomnia, vertigo (mediated by the central nervous system) and nausea, and vomiting (mediated by the gastrointestinal system). These adverse drug reactions were transient and were resolved. The results suggest that nalfurafine seem to be both an effective and safe drug in the treatment of patients with ESRD undergoing hemodialysis.

Questionnaires for Itch Assessment

Yosipovitch et al. constructed two questionnaires for itch assessment modifying McGill's pain questionnaire (20,21). The first study utilized a predetermined questionnaire that provided a detailed description of pruritus in AD in 100 atopic Chinese patients (20). The modified questions were aimed to characterize the clinical pattern and sensory and affective dimensions of itch experience in AD. Itch intensity was also measured using VAS. Results revealed: (*i*) prolonged duration of pruritus (descending order) in lower limbs, flexures, upper limbs, and neck; (*ii*) itch intensity peaked twice as much as mosquito-bite itch; (*iii*) itching was most frequent at night, and most patients reported difficulty in falling asleep; (*iv*) daily-life activities that increased severity of the itch were (descending order) sweat, dryness, stress, physical effort, specific fabrics, activity, and hot water. Males significantly ($p = 0.004$) differed with females in terms of activity and physical effort ($p = 0.002$) in increased pruritus; (*v*) major factors found to reduce itch included bathing in cold water and cold ambient environment; (*vi*) associated symptoms were heat sensation, sweating, and pain in the pruritic area; (*vii*) most antipruritic medications have limited long-term effects; (*viii*) itch is bothersome and a major distress to the patient; and (*ix*) the affective score significantly ($p < 0.001$) correlated to itch intensity during its peak. Taken together, the questionnaire was found to be a useful tool in characterizing itch.

The other questionnaire constructed to measure pruritus was based on the short form of the McGill Pain Questionnaire in 145 uremic patients (21). This modified questionnaire included (*i*) patient characteristics; (*ii*) the use of antipruritics; (*iii*) effects of pruritus on sleeping and on mood; (*iv*) effects of dialysis and of daily activities on itch; (*v*) location pruritic sites; (*vi*) sensory and affective scores; and (*vii*) itch intensity measured using VAS. Revalidation of the questionnaire was repeated in 28 subjects after two weeks and revealed no significant ($p > 0.05$) difference in VAS temporal states (onset, pattern, course) and no significant ($p > 0.05$) difference with regard to the sites of the itch between the two questionnaires. Also, the reliability was high ($p < 0.01$).

Studies of Possible Models for Establishing New Antipruritics

As mentioned earlier, Orito et al. developed a model for itch assessment using the SCLABA system and proposed what may be a potential model for development of new antipruritics (8).

Thomsen et al. also proposed two models that could benefit in developing new antipruritics (10,12). The first animal model is to topically apply nonhistaminic antipruritics using serotonin, recognized as a weak local pruritogen in humans (12). Out of the eight

Table 1 Summary Data of Models and Efficacy of Antipruritics

Models	Efficacy	Reference
Intradermal histamine injection-induced itch	5-fold increased above baseline of the histamine dose required, producing pruritus following both cyproheptadine and placebo. A 10-fold increased following diphenhydramine and a 750-fold increase following hydroxyzine HCl.	Rhoades et al. (22)
Intradermal histamine injection-induced itch	Clobetasol propionate ointment rapidly decreased itch but did not alter warmth sensation and thermal pain thresholds.	Yosipovitch et al. (23)
Intradermal histamine injection-induced itch	Menthol failed to show the effect of antipruritics.	Yosipovitch et al. (24)
Intradermal histamine injection-induced itch	Aspirin significantly reduced itch duration and decreased itch magnitude.	Yosipovitch et al. (25)
Intradermal histamine injection-induced itch	Pramoxine significant reduced both the magnitude and duration of itch.	Yosipovitch et al. (26)
Histamine iontophoresis-induced itch	Capsaicin significantly reduced itch sensations.	Weisshaar et al. (28)
Intradermal histamine injection-induced itch	Aspirin did not decrease histamine-induced itch.	Thomsen et al. (29)
Intradermal histamine injection-induced itch	2.5% hydrocortisone significantly reduced histamine-induced itch.	Zhai et al. (31)
Intradermal histamine injection-induced itch	Strontium nitrate showed a good antipruritic effect in reducing histamine-mediated itch.	Zhai et al. (30)
Intradermal histamine injection-induced itch	Pramoxine-containing cream was the most effective antipruritic in decreasing histamine-induced itch.	Zhai et al. (32)
Histamine iontophoresis-induced itch	Noxious heat, noxious cold, and scratching attenuated itch via a spinal or supraspinal mechanism.	Yosipovitch et al. (14)
Histamine iontophoresis-induced itch	Heat pain and scratching reduced itch.	Yosipovitch et al. (15)
Histamine iontophoresis-induced itch	Ear electro-acupuncture reduced alloknesis areas on the forearms.	Kesting et al. (18)
Skin-pricked-histamine-induced itch	A decrease in short-term temperature enhances histamine-induced-itch.	Pfab et al. (16)
Intradermal injection of 8 pruritogens	Histamine and substance P were more pruritogenic in SLS-induced inflamed skin.	Thomsen et al. (10)
Scratch behavior measurement	Byakko-ka-ninjin-to significantly inhibited the scratching frequency in NC mice.	Tohda et al. (33)
Intradermal serotonin induced-itch	Serotonin is a reproducible pruritogen eliciting scratch behavior in rats.	Thomsen et al. (12)
Intradermal compound 48/80-induced itch	MicroAct was comparable with the observer's tally in scratching behavior in mice.	Inagi et al. (9)
Intradermal compound 48/80-induced itch	SCLABA image analysis system was as good as the "true" scratching behavior in mice.	Orito et al. (8)
Intradermal substance P–induced itch	Sertconazole nitrate was comparable with 1% hydrocortisone in reduction of scratching behavior of mice.	Liebel et al. (11)
Lesional skin of atopic patients	Limb-worn accelerometers were comparable with night video-recording in assessing scratch behavior.	Benjamin et al. (17)
Uremia-induced itch	K-opioid agonist nalfurafine was effective and safe treatment in patients with uremia.	Wikström et al. (19)
Predetermined questionnaire (Modified McGill Pain Questionnaire)	Predetermined questionnaire was useful in the assessment of itch in atopic dermatitis patients.	Yosipovitch et al. (21)
Modified McGill Pain Questionnaire	The reliability of Modified McGill Pain Questionnaire was high when compared with the VAS results.	Yosipovitch et al. (20)

substances screened (histamine, compound 48/80, kallikrein, trypsin, papain, substance P, serotonin, and platelet-activating factor) injected intradermally (50 μL per substance) into the rostral back of rats, only serotonin induced excessive scratching, while the rest of the substances were weak or inactive. A dose-response curve was plotted against $\log_{10}$ using different

concentrations of serotonin to evaluate possible systemic effects in: (*i*) 14 rats intradermally and subcutaneously injected with 0.1 and 1 mg/mL (50 μL per dose) to the rostral and caudal back; (*ii*) another four rats were given intradermal serotonin of 10 mg/mL to the caudal back; (*iii*) another 10 rats for each group were given concentrations of 0.01 to 31.6 mg/mL intradermally; and (*iv*) two rats were given concentration of 100 mg/mL each. Video recording was used to objectively count scratch sequences, viewed separately by two investigators, and showed the following: (*i*) number of scratch sequences of injected serotonin related to the rostral back is significantly greater ($p < 0.001$) than that of the caudal back; and (*ii*) number of scratch sequences in the caudal and "other" sites did not produce a significant result. No systemic adverse effects of serotonin occurred at 1 mg/mL, 50 μL. Scratching was probably not due to histamine, since the screening period revealed that histamine did not produce scratching. They concluded that serotonin is a reproducible pruritogen eliciting scratch in rats.

The second study was a randomized, double-blind and placebo-controlled study involving SLS-induced inflamed skin, as well as in normal skin in 32 healthy volunteers, pretreated with 1% SLS in one of their volar forearms, their opposite forearms served as control (10). They evaluated itch intensity, pain, whealing, and redness in 16 subjects given 20 μL of group A battery of substances [substance P, neurokinin A, neurokinin B, histamine (positive control), and physiological saline (negative control)], and the other 16 given were given 20 μL of group B battery of substances [platelet-activating factor, serotonin, trypsin, histamine (positive control), and physiological saline (negative control)], all intradermally injected to both forearms. Results revealed inflamed skin is significantly more pruritogenic than normal skin in substance P ($p = 0.024$) and histamine compared with the control. Neurokinin A, trypsin, PAF, and serotonin only elicited itch in normal skin, while neurokinin B did not elicit itch in both groups. Wheal area was significantly ($p < 0.001$) larger in inflamed skin, though it did not show a significant correlation with itch intensity (Table 1).

CONCLUSION

When measuring itch, several factors must be taken into account: severity, duration, variation between individuals, and subjective differences in determination of itching threshold. To better quantify this subjective response, several approaches have been employed (11–19,27,34–38). The measurement of scratch behavior is problematic, and has been addressed in a variety of ways such as lack of validation and unlikeliness to be reproducible, etc. (12). Since human's verbalization may be more accurate in describing itch sensation, the VAS may provide superior to other methods (11,12,18,19). To evaluate antipruritic drugs, clinical methods may rely on either naturally occurring or experimentally induced pruritus. Methods and judgments based on naturally occurring pruritus better reflects the actually clinical setting (11). However, they have disadvantages including: (*i*) pruritus intensity may fluctuate on its own if the study is conducted over several days since the naturally occurring pruritus may not be stable over time; (*ii*) comparing the pruritic intensity of specific lesions in different patients is often difficult and not always relevant, and (*iii*) adequate controls are difficult to achieve (11).

Histamine-induced itch model was utilized because acute itching is most commonly evoked by chemical stimuli (e.g., histamines) (2). Some individuals do not itch after histamine injection (34,35,38); therefore, to diminish the variation of responses, we suggest that only subjects with histamine-induced itch sensation should be enrolled. This will improve discrimination–an obvious advantage in a screening assay. However, the histamine injection model may induce pain sensation; it may partially interrupt the itch sensation. We note that 1 mL injection appears high and undoubtedly spreads; this large volume has added reproducibly to previous studies (16,17,27,34–38). The VAS score was comparatively low; however, this level (3 cm) was adequate for the discrimination noted. Higher concentrations might be considered in the future.

Alternatively, other itch-inducing models may well be justified in the assessment of antipruritic drugs, complemented by new measuring devices (8–12,14–18,27,39). A recent review of pruritus provides additional insights (40).

Lastly, it is essential that studies of topical antipruritics are well designed and double blind so that resulting data are valid and able to distinguish between effective and noneffective treatments.

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56 Comedogenicity in Rabbit: Some Cosmetic Ingredients/Vehicles

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INTRODUCTION

Several cosmetic ingredients have been shown to be comedogenic using the rabbit ear assay (1,2). On the basis of the animal assay and short-term human clinical studies, the development of comedones has been attributed to prolonged use of cosmetics (1,2). Although there are limitations of the rabbit model in the application of test results to humans (1–4), much information on the comedogenicity of topically applied substances has been based on the rabbit ear assay because it permits rapid screening of many possible offenders. The present chapter is concerned with this theme and reports our finding on the activity of certain raw materials previously investigated.

MATERIALS AND METHODS

To quantify the rabbit comedogenicity data obtained using Kligman's design (1) for acnegenic potential of coded samples in the rabbit ear assay, test and control articles were purchased commercially, which included stearyl alcohol; sodium lauryl sulfate (SLS)—0.1%, 1.0%, 10.0% in petrolatum; butyl stearate—1.0%, 10.0%, 25.0% in petrolatum; isopropyl palmitate 1.0%, 10.0%, 25.0% in petrolatum; myristyl myristate (50% in petrolatum); isopropyl myristate (50% in cold cream); isopropyl palmitate (50% in vanishing cream); isopropyl myristate (50% in vanishing cream); isopropyl myristate (50% in propylene glycol); isopropyl palmitate (50% in propylene glycol); and isopropyl myristate (50% in ethanol). Isopropyl palmitate (δ prime); butyl stearate; paraffin; cetyl alcohol; cocoa butter; decyl oleate; isostearyl neopentonate; isopropyl isostearate; isocetyl stearate; commercial cocoa samples labeled A, B, C, D, E, F, and G; guittard cocoa butter (control); and petrolatum were tested as is.

Samples were stored at room temperature. All test samples were of USP grade.

New Zealand male and female white rabbits, randomly outbred, were delivered at 2.5 kg and acclimated at least four days before entering the study. The rabbit was selected as the test system because of its proclivity to develop comedones (1–6).

Animal identification was via tattoo. The rabbits kept on an 11- to 12-hour light/dark cycle, at room temperature, 71°F to 74°F (22–23°C), and one per cage, were fed commercial laboratory feed (purina chow) and treated potable water available at all times.

The cosmetic ingredient samples were applied to the ears of adult female albino rabbits according to the Rabbit Ear Comedogenic Assay. Each sample was applied daily (Monday through Friday) to the glabrous inner portion of three ears for four consecutive weeks, adding up to a total of 20 applications. Approximately, 0.5 mL or 0.5 gm of the sample was applied with a pipette or syringe, and spread with a glass applicator. Controls consisted of 10% crude coal tar. Coal tar evoked a comedogenic score of 4 on all control animals (data not shown). Ingredient samples were applied to both ears during the tests. At the end of four weeks, each rabbit ear was biopsied and examined for evidence of comedone formation. An elliptical sample, about 2.5 cm long, was blunt dissected down to the cartilage and immersed in water at 60°C for two minutes. The epidermis was peeled off as an intact sheet; its undersurface

examined under a stereomicroscope. Grading was done in a manner similar to the visual grading system (see below).

Scoring System: 0 = none

1 = a few comedones
2 = many comedones
3 = extensive comedones
4 = confluent involvement

RESULTS

Test results (Table 1) indicate that the ester ingredients selected had comedogenic scores that ranged from 1 to 4: isopropyl palmitate, butyl stearate, isopropyl isostearate, and decyl oleate scored highest in its pure form with scores ranging from 3 to 4. Manipulation of testing conditions through dilution of concentration and mixture of ingredients either increased or decreased comedogenic potentials. The addition of vanishing cream to isopropyl palmitate and isopropyl myristate increased their comedogenicities, pushing means scores close to 4, despite a 50% dilution. Isocetyl stearate, myristle myristate, cetyl alcohol, and stearyl alcohol had the least comedogenic potential, with mean scores of only 1 to 2.

SLS was tested in petrolatum at concentrations of 0.1%, 1%, and 10%. Results (Table 2) indicate low comedogenic potentials for these specific combinations. Mean scores were in the range of 1.

Table 1 Comedogenic Scores of Esters and Alcohols

	Mean day 20 (clinical)		Mean (slide biopsy)	
Test article	L	R	L	R
Isopropyl palmitate (δ prime)	3 ± 0	3.6 ± 0.5	3.6 ± 0.5	3.6 ± 0.5
Isopropyl palmitate 1% in petrolatum	1.3 ± 0.5	1.6 ± 0.5	1.3 ± 0.5	1.6 ± 0.5
Isopropyl palmitate 10% in petrolatum	1.3 ± 0.5	1.3 ± 0.5	1.3 ± 0.5	1.6 ± 0.5
Isopropyl palmitate 25% in petrolatum	1 ± 0	1.6 ± 0.5	1 ± 0	1.6 ± 0.5
Isopropyl palmitate in vanishing cream (50%)	4 ± 0	4 ± 0	3.6 ± 0.5	3.6 ± 0.5
Isopropyl palmitate in propylene glycol (50%)	3 ± 0	3 ± 0	3 ± 0	3 ± 0
Isopropyl myristate in cold cream (50%)	3 ± 1	3.3 ± 0.5	3 ± 1	3.3 ± 0.5
Isopropyl myristate in vanishing cream (50%)	4 ± 0	4 ± 0	4 ± 0	4 ± 0
Isopropyl myristate in propylene glycol (50%)	3 ± 1	3 ± 1	3.3 ± 0.5	3.3 ± 0.5
Isopropyl myristate in ethanol (50%)	4 ± 0	4 ± 0	4 ± 0	4 ± 0
Butyl stearate	4 ± 0	4 ± 0	4 ± 0	4 ± 0
Butyl stearate 1% in petrolatum	1 ± 0	1 ± 0	1 ± 0	1.3 ± 0.5
Butyl stearate 10% in petrolatum	1.6 ± 0.5	2 ± 0	2 ± 0	2 ± 0
Butyl stearate 25% in petrolatum	2 ± 0	2 ± 0	2 ± 0	2 ± 0
Isopropyl isostearate	3.6 ± 0.5	4 ± 0	3.6 ± 0.5	4 ± 0
Decyl oleate	3.3 ± 0.5	3.3 ± 0.5	3.3 ± 0.5	3.3 ± 0.5
Isostearyl neopentanoate	2 ± 0	2 ± 0	3 ± 0	3 ± 0
Isocetyl stearate	1 ± 0	1 ± 0	2 ± 0	2 ± 0
Myristle myristate	1.3 ± 0.5	1.3 ± 0.5	1.6 ± 0.5	2 ± 0
Cetyl alcohol	1.3 ± 0.5	1.3 ± 0.5	1 ± 0	1.3 ± 0.5
Stearyl alcohol	1.3 ± 0.5	1.3 ± 0.5	1 ± 0	1 ± 0

Table 2 Comedogenic Scores of Surfactants

	Mean day 20 (clinical)		Mean (slide biopsy)	
Test article	L	R	L	R
SLS 0.1% in petrolatum	1 ± 0	1 ± 0	1 ± 0	1 ± 0
SLS 1% in petrolatum	1 ± 0	1.3 ± 0.5	1 ± 0	1 ± 0
SLS 10% in petrolatum	1.6 ± 0.5	1.6 ± 0.5	1.3 ± 0.5	1.3 ± 0.5

Abbreviation: SLS, sodium lauryl sulfate.

Table 3 Comedogenic Scores of Petrolatum Products and Cocoa Butter

	Mean day 20 (clinical)		Mean (slide biopsy)	
Test article	L	R	L	R
Petrolatum (control)	1 ± 0	1.3 ± 0.5	1 ± 0	1.3 ± 0.5
Paraffin	1 ± 0	1.3 ± 0.5	1 ± 0	1 ± 0
Cocoa butter (#81-C FDA-1B)	3.3 ± 0.5	3.3 ± 0.5	3.3 ± 0.5	3.6 ± 0.5
Cocoa butter (#82-C FDA-2)	3 ± 0	3 ± 0	3.3 ± 0.5	3 ± 0
Cocoa butter A	2.6 ± 0.5	3.3 ± 0.5	2.6 ± 0.5	2.6 ± 0.5
Cocoa butter B	3.3 ± 0.5	3.6 ± 0.5	3.3 ± 0.5	3.3 ± 0.5
Cocoa butter C	3 ± 0	3 ± 0	2.6 ± 0.5	2.6 ± 0.5
Cocoa butter D	3.6 ± 0.5	4 ± 0	4 ± 0	4 ± 0
Cocoa butter E	3.6 ± 0.5	4 ± 0	3.6 ± 0.5	3.6 ± 0.5
Cocoa butter F	3.6 ± 0.5	3.6 ± 0.5	3.6 ± 0.5	3.6 ± 0.5
Cocoa butter G	3.3 ± 0.5	3.6 ± 0.5	3.6 ± 0.5	3.6 ± 0.5
Cocoa butter (control)	3 ± 0	3 ± 0	3.3 ± 0.5	3 ± 0

Tables display comedogenic potential scores ± standard deviation based on a 5-point scale, in which 4 is the highest score. Results listed were obtained at the end of the four-week test period (day 20) using visual and steromicroscopic (slide biopsy) examination. The cocoa butter samples represented different commercial production batches.

Paraffin and petrolatum, used as control vehicles, did not yield significant comedogenic potential scores, whereas cocoa butter displayed high results (Table 3). The majority of comedogenic scores fell between 3 and 4.

DISCUSSION

Among the tested ingredients, decyl oleate, isopropyl palmitate, isopropyl myristate, isopropyl isostearate, isostearyl neopentanoate, isocetyl stearate, myristyl myristate, butyl stearate, and cocoa butter were deemed comedogenic. All of the esters are most commonly found in products such as night cream, wrinkle removal cream, sunscreen, moisturizer, hair care products, lipstick, concealer, antiperspirant, as well as baby care products. Cocoa butter is a prevalent ingredient in cosmetics due to its smooth texture and sweet fragrance. It is found in soaps, lotions, skin care products, and suntan lotion. Since we rely on the use of common household cosmetics to maintain our hygienic regimen, comedogenicity should be considered in the development of cosmetics, skin care products, and topical medications. The Cosmetic, Toiletry, and Fragrance Association (CTFA), the Cosmetic Ingredient Review (CIR), and physicians may consider providing available information about the ingredients of such products to their consumers. These findings are significant and are worth noting; otherwise, consumers may be misguided about the effects of using such products (7).

Data from this study generates a theory for dosage and purity dependence. The initial tests for isopropyl palmitate and butyl stearate were done using the ingredients in their purity at 100% concentration, yielding a mean score of 3.3 and 4, respectively, for week 4. However, when the tests were repeated using isopropyl palmitate and butyl stearate at concentrations of 1%, 10%, and 25% in conjunction with petrolatum, results were significantly different. Scores declined to an average of 1 to 1.7 for isopropyl palmitate and an average of 1 to 2 for butyl stearate at week 4. The results indicate that the comedogenic properties of these ingredients depend on either dosage or purity. Unfortunately, since both purity and concentration were manipulated in one test, it is not possible to determine whether the reduction in the comedogenicity was due to the dilution of concentration, purity, or both. In another case where vanishing cream was added to isopropyl palmitate and isopropyl myristate, comedogenicity escalated to a score of 4, despite a 50% dilution. Is it possible that cosmetic products developed using recommended USP grade have potent acnegenic results? This is a matter that may be worth investigating.

The ultrasensitivity of the rabbit follicle to respond readily to test materials has been extensively documented (1–6,8). Our study sought to quantify comedogenic data collected with the commonly used model: Kligman's rabbit assay. However, the lack of a systematic

database for such information restricts the use of compiled results as a direct correlation to humans. Instead, results from such studies should only be used as a guideline for formulation programs.

To further correlate these results with human reactions, specific studies on humans need to be done. Kligman's four-week patch test conducted on human subjects demonstrates a positive correlation between the animal and human model (4). However, there are two major differences worth noting. First, humans are less sensitive to "acne cosmetica" than rabbits (4). For example, comedogenic substances of scores 1 or 2 that evoke comedones on rabbits may not affect humans (4). Second, the rabbit model takes longer to yield results than the human model. Humans usually experience acute pustulation during the first 24 hours, whereas rabbits typically take three to four weeks to fully express comedones (9). Comedone expression is also present in humans, but it does not occur as frequently as on rabbits. These differences suggest that there may be a different mechanism involved in the production of pustules on humans.

Taken together, this data may be added to dermatotoxicologic ingredient profiles; yet much remains to be done before we can fully comprehend its meaning. Results obtained by Fulton (10) have deemed isopropyl myristate, isopropyl palmitate, isopropyl isostearate, butyl stearate, isostearyl neopentanoate, myristle myristate, decyl oleate, and isocetyl stearate as offenders. However, the CTFA and CIR maintains that the use of all these ingredients is safe at its appropriate concentration (11).

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