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Skin, drug and chemical reactions

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Several drugs and chemicals applied to the skin result in some local reaction by the skin to the applied compound or its formulation. These reactions may range from generalized reactions as characterized by an adaptive immune or allergic response to a specific reaction such as to light to a localized alteration in the barrier properties of the outermost physical skin barrier, the stratum corneum. This overview considers the mechanisms by which the skin reacts to various chemicals it becomes exposed to, giving some examples of how the interactions can occur, the compounds involved, and chemical structure active relationships associated with the interaction between specific compounds and the skin.

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Introduction

The skin plays a major role in acting as a physical, immune and sensing barrier to chemicals in our environment that we may become exposed to. Such chemicals include those that may be absorbed into the skin accidentally after environmental, occupational or recreational exposure or, in the case of a cosmetic or dermatological, applied to the skin deliberately. In this overview, we limit our considerations to examples of chemical reactions with the skin most relevant to those working in drug discovery. We highlight first those compounds which could cause allergic contact dermatitis and their mechanisms. We then look at those compounds which lead to phototoxic and photoallergic reactions. This is then followed by a study of chemical structure activity relationships associated with those processes. Finally, we examine the local effects of topical products on the outermost physical barrier layer of the skin, the stratum corneum, with its possible sequelae of corrosion and direct irritation. A more detailed overview on each of these aspects has recently been published [1]. That overview goes beyond the considerations here in also considering the role of skin morphology in dermal skin absorption, cutaneous metabolism, role of blood flow and lymphatics, the modelling of skin penetration, structure activity relationship for skin corrosivity and sensitization, photosensitization and regulatory aspects.

Mechanisms of allergic contact dermatitis

Allergic contact dermatitis (ACD) is an adaptive immune response towards chemical allergens penetrating the skin. ACD progresses in two phases: (1) in the initial sensitization phase the host is immunized to the allergen, and (2) in the elicitation phase, a rapid secondary immune response is mounted following re-exposure to the allergen. The elicitation phase manifests as ACD.

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Initiation of inflammation – sensitization phase

The typical contact allergen is a small and reactive molecule which also has irritant capacities. Contact allergens penetrate the upper layers of the skin and interact with skin proteins forming hapten-carrier protein complexes. Hapten-complexes are internalized by immature dendritic cells (DCs) [2] which are efficient at antigen internalization, but quite inefficient at antigen presentation. Contact allergen-induced irritation activates innate immunity system (Fig. 1), causing skin cells to secrete proinflammatory cytokines (e.g. TNF-α, IL-1-β) which are important for the maturation of DCs. During maturation, the cell surface expression of antigen surface receptors on the DC is decreased and, at the same time, surface expression of MHC molecules and T-cell co-stimulatory molecules increases [3]. Maturation of DCs also induces modification in the expression of chemokine receptors and adhesion molecules which critically contribute to the migration of DCs to secondary lymph organs in which initiation of adaptive immunity will take place.

Immature DCs express receptors for inflammatory chemokines (e.g. CXCR1, CCR1, CCR2, and CCR5). As DCs mature, they lose their responsiveness to inflammatory chemokines through receptor downregulation or desensitization, but acquire responsiveness to CCL19 and CCL21 as a consequence of CCR7 up-regulation. CCR7 has a prominent role in driving DC migration to the lymph nodes [4–6]. DC migration is central to CHS initiation, and mice deficient in CCR7 [7] have their CHS responses suppressed. Fully mature DCs produce chemokines such as CCL18 and CCL19, which attract naive T-cells [8]. During the initial encounter between the DC and the T-cell, adhesion molecules DC-SIGN and ICAM-1 interact with ICAM-3 and CD11a/CD18 [9].

Presentation of antigens

DCs process engulfed antigens into peptide fragments, and load them onto major histocompatibility (MHC) molecules. MHC class I is recognized by the CD8 co-receptor on cytotoxic T-cells, and MHC class II by the CD4 co-receptor on helper T-cells [3]. A third class of MHC molecules, the CD1d molecule is complexed with a synthetic lipid antigen, a-galactosylceramide (aGalCer), and activates natural killer (NKT) T-cells [10]. NKT cell-deficient mice (Ja18−/−, CD1d−/−) fail to elicit CHS responses suggesting that these cells have an essential role in CHS reactions [11].

In addition to TCR ligation, T-cells need co-stimulatory signal for proper activation [12]. In the best-characterized pathway CD28 receptor binds to co-stimulatory molecules CD80 and CD86. Signalling via the CD28 receptor promotes expansion of antigen-stimulated T-cells and their differentiation into effector and memory cells. The CD28-related protein, CTLA-4, is constitutively expressed on the surface of T regulatory cells, and is induced on activated T-cells. Contrary to CD28, CTLA-4 delivers an inhibitory signal to the activated T-cells. A third CD28 related protein ICOS is induced on activated T-cells, and binds its ligand LICOS on activated DCs, monocytes and B cells. The role of LICOS needs to be defined in further studies.

The activated T-cell also expresses several proteins which regulate co-stimulatory signalling. Interaction between CD40 ligand on T-cells and CD40 on DCs increases the expression of CD80 and CD86 molecules which further intensifies the interaction between T-cells and APC [13]. Moreover, OX40 (CD134), 4-1BBL, CD70 and LIGHT also provide co-stimulation upon TCR stimulation, leading to full and effective T-cell responses [14].

Elicitation phase – leukocyte recruitment

In individuals previously sensitized to hapten, the next encounter with the same hapten results in an elicitation phase that manifests as ACD. The clinical signs of ACD, including heat, oedema, itching and vesicles, are caused by leukocyte infiltration in the skin one to two days after the onset of the elicitation phase. The infiltrates are formed by leukocytes arriving from the blood circulation. The interaction between selectins and their carbohydrate ligands cause leukocytes to roll onto the vascular endothelium. Thereafter, chemokine receptors on leukocytes are exposed to their ligands on endothelial cells leading to the rapid activation of integrins which results in firm adhesion and transmigration [5].

In addition to αβ T-cells, NKT cells, B1-cells and γδ T-cells are also essential to the CHS response [15–17]. Glycolipid ligands activate NKT cells start to secrete IL-4 which then co-activates B-1 cells. B-1 cells produce hapten-specific IgM antibodies that enter the circulation. At the elicitation phase, local B-1 cell-derived IgM forms complexes with allergens, which activates complement 5, and the subsequent generation of C5a. Thereafter, C5a activates C5a receptors on mast cells and platelets to induce release of TNF-α, and serotonin, which cause recruitment of effector αβ T-cells. The γδ T-cells are a helper cell subset for effector αβ T-cells for optimization of the response [18]. Finally, the effector αβ T-cells attract leukocyte infiltrates of ACD [19]. The relative importance of these subsets of leukocytes has been supported by the findings that mice deficient in NKT cells [10], B-1 cells [20], complement C5 [21] and C5 receptors [22] have all impaired CHS.

Strong evidence exists that CHS responses to haptens are mediated by CD8+ T-cells whereas the role of CD4+ T-cells remains less clear. However, it has been reported that CHS reaction is impaired in CD4-deficient mice (CD4−/−) [23,24]. Cytotoxic function of CD8 T-cells, on the other hand, is impaired in CD4 deficient mice [25].

Th1 cells predominate in ACD but also Th2 cells have been isolated from human ACD lesions, although their role is still

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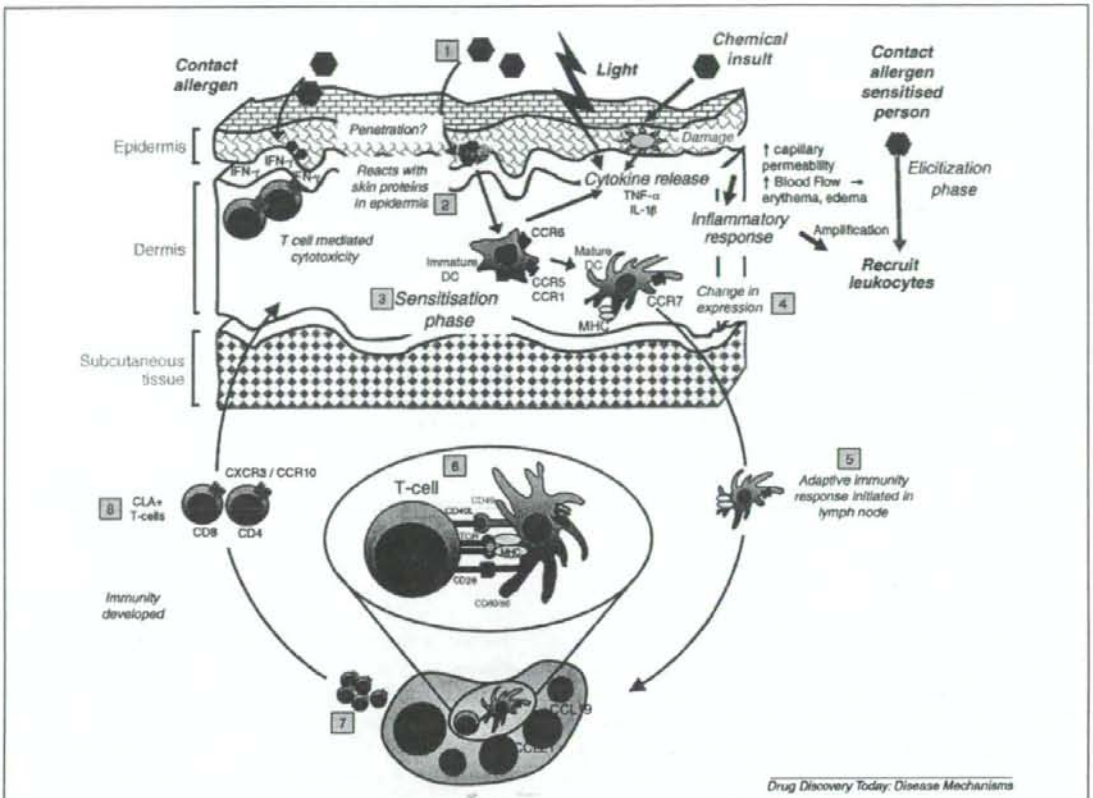

Figure 1. Model for sensitization (steps 1–7) and elicitation (steps 8–9) phase in ACD . Haptens (○) penetrate epidermis (1) and interact with skin proteins forming hapten-carrier protein complexes (2). Hapten-complexes are internalized by immature DCs (3). Contact allergen induced irritation induces secretion of proinflammatory cytokines which are important for the maturation of DCs. During maturation (4), the expression of receptors for inflammatory chemokines is decreased whereas expression of MHC molecules and chemokine receptor 7 (CCR7) is enhanced (5). Initiation of adaptive immune responses will take a place in draining lymph node in which processed antigen is presented in the context of MHC molecules to naive T-cells (6). In addition to TCR ligation, T-cells need co-stimulatory signal (6) (e.g. CD28–CD80/86 and CD40–CD40L interaction) for proper activation and proliferation of antigen specific T-cells (7). The second encounter to the same hapten results in recruitment of allergen specific T-cells (CD4+ or CD8+ T-cells bearing CLA, as well as CXCR3 and CCR10 receptor in their surface) (8) to the site of allergen exposure. Hapten specific T-cells secrete IFN-γ and elicit T-cell mediated cytotoxicity resulting in inflammation which manifests as ACD (9).

unclear [26]. Mice lacking IL-4, a major Th2 cytokine, have impaired CHS responses [27]. Interleukin-17 (IL-17) is a cytokine that is expressed by a new subtype of T-cells, the Th17 cells. Mice lacking the IL-17 gene demonstrate impaired CHS reactions, suggesting a role for IL-17 in ACD [28].

IFN-γ-inducible chemokines CXCL10, CXCL9 and CCL5 are important in Th1 inflammation [5]. Knockout mice without CXCL10, a ligand for CXCR3, do have an impaired CHS response. Keratinocytes produce CCL27 which is the ligand of CCR10, expressed on skin-specific CLA+ effector or memory T-cells. Neutralization of the interaction CCL27 and CCR10 results in impairment of CHS [29]. Th1 cells, monocytes and macrophages express CCR5 on their surface. The relative importance of CCR5 in Th1 reactions is not yet clear, but mice deficient in CCR5 have enhanced CHS responses [30], suggesting a downmodulating role for CCR5. CCR6, that is involved in the recruitment of CD4+ IL-10 producing T regulatory cells, has a suppressive effect, too, as in CCR6 deficient mice CHS is enhanced and persistent [31]. DCs, following activation, produce inflammatory chemokines CCL3, CCL4 and CXCL8, followed by CCL5 and CCL2 which contribute to the leukocyte recruitment.

Downregulation of contact dermatitis

Duration and strength of the ACD reaction is regulated by T regulatory cells (Tregs). Tregs mediate a suppressive effect through inhibiting the activity of T-cells [32], DCs [26], B-cells, basophils and eosinophils [33] with partially unknown

mechanism. However some Treg subtypes seem to act through cell contact, and others through the release of anti-inflammatory cytokines such as IL-10 and TGF-β [32]

When the inflammation in ACD is terminating, the activated T-cells undergo programmed cell death, that is apoptosis. FAS (on T-cells)-FAS ligand (e.g. on DCs) interaction is the best-described apoptosis pathway. Cytotoxic molecules such as perforin, granzyme B and FAS ligand are also crucial for the expression of CHS. Mice deficient in perforin and Fas ligand fail to mount CHS reactions to haptens [34].

Phototoxic and photoallergic reactions

Both systemic and topical exogenous photosensitizers evoke cutaneous photosensitivity, and clinically recognized as drug photosensitivity and photocontact dermatitis, respectively. The action spectrum of these two types of photosensitivity is mainly UVA [35], and UVB rarely evokes the diseases, as represented by photosensitivity to sulfanilamide and ranitidine. Photoaugmentation between UVA and UVB occasionally occurs as in sparfloxacin [36].

Photosensitive chemicals have both phototoxic and photoallergic potentials. Phototoxic reaction is mainly mediated by free radicals and eventually results in a cellular cytotoxicity, whilst photoallergic reaction is induced and elicited by immunological consequences involving various immunocompetent cells and molecules [37,38]. Each photosensitive chemical has different dominancy to phototoxicity or photoallergenicity. For example, psoralen and porphyrin derivatives are strong phototoxic agents with scarce photoallergenicity, and thus employed for photochemotherapy or photodynamic therapy with little photoallergic adverse effect. By contrast, fluoroquinolones and halogenated salicylanilides are causative agents for photoallergic drug eruption and photocontact dermatitis, respectively [38,39]. It is noted, however, that all photoallergic chemicals have a various degree of phototoxic property, because the photoallergic reaction needs the phototoxic step where photosensitizers bind to protein via the formation of free radicals [35]. Historically, it had been believed that most cases of drug photoallergy and photocontact dermatitis are induced by phototoxic reaction, and the incidence of photoallergic type is low. However, recent clinical studies have suggested that the photoallergic type is higher than the phototoxic type in incidence [37]. This misunderstanding seems to be caused by easy evaluation of phototoxicity and difficult assessment of photoallergenicity.

Phototoxicity is mainly caused by the generation of oxygen intermediates [36,39]. Among them, singlet oxygen is most important for drug phototoxicity and generally termed type II reaction [39]. The target molecules of phototoxic chemicals include proteins or amino acids, lipids, and DNA [36,39], and their alterations lead to cellular damage or even cellular death. Therefore, cellular cytotoxicity has been used as a classical method to evaluate phototoxicity. Both necrosis and apoptosis occur in cells phototreated with chemicals and UV [40]. Various cells have been utilized for cytotoxicity assessment, including erythrocytes, fibroplasts, keratinocytes, macrophages, lymphocytes, and even fungi, but the reduction of neutral red uptake in phototreated 3T3 fibroblasts may become the standard assessment. Phototoxicity also can be evaluated by using target molecules, and such tests include protein (histidine) degradation, lipid oxidation, and plasmid DNA breaking activity [36]. In addition, the binding capacity of chemicals to protein upon exposure to UV is a phototoxicity test [41]. Although this reaction is derived from phototoxic moiety of chemicals, the resultant chemical–protein complex affords a photoantigenic determinant. Thus, it is recently considered that photobinding of agents with protein represents a photoallergic potency of a given chemical.

Photoallergenicity is a well-organized immunological reaction. The current understanding of ordinary contact dermatitis and drug hypersensitivity is on the basis of the hapten hypothesis: they bind covalently to proteins, and the resulting conjugates can be recognized as immunogenic determinants. Likewise, photosensitive chemicals have a haptenic moiety. As illustrated in Fig. 2, two hypotheses have been put forward to explain the formation of photoallergen. One is that the photosensitizer is a prohapten, which is converted to a complete hapten by UV irradiation, and the resultant hapten can bind to protein. In another theory, the photosensitizer is a photohapten, which needs to coexist with protein, and upon UV irradiation, a covalent bond takes place via the formation of free radicals. In the case of photohaptens, therefore, UVA-preirradiated photosensitive chemicals are incapable of binding to protein. In clinical photopatch test, a causative chemical is applied to the skin and UVA is irradiated to the same site. This method is for

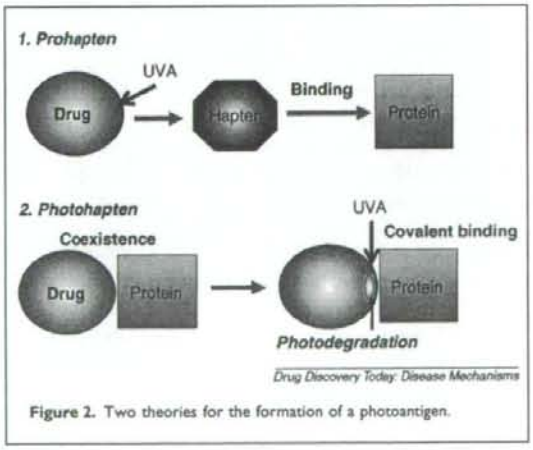

Figure 2. Two theories for the formation of a photoantigen.

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testing the photohaptenic property. In the case of prohapten, however, an UVA-preirradiated chemical should be applied to the skin as patch test. Empirically, a photopatch test has been performed to test photoallergy. The vast majority of clinically photoallergic chemicals are photohaptens rather than prohaptens [35,41,42]. Accordingly, patients usually exhibit a positive photopatch test to culprit chemicals but a negative patch test to UVA-preirradiated chemicals.

UVA is the action spectrum of photoderivatization of proteins or cells with photoallergic chemicals [41,42]. The mechanisms underlying photoallergic contact dermatitis are virtually the same as those of ordinary contact dermatitis except for the requirement of UV irradiation in sensitization and challenge [38]. Photocoupling of epidermal cells with a photocontactant is the initial step for this immunological response. Similarly, in drug photoallergy, an orally administered drug diffuses from the blood to the epidermis, and epidermal cells are photoderivatized with the drug upon UVA irradiation [43]. Among epidermal cells, antigen-presenting dendritic cells, that is Langerhans cells are critical, which are photomodified with a photocontactant or a systemically given drug upon UVA exposure to the skin and become photohapten-bearing, T-cell stimulatory cells [44]. Causative photohaptens are bound to MHC class II molecules/self peptide on Langerhans cells upon exposure to UVA [45]. The photomodified Langerhans cells sensitize and elicit antigen-specific T-cells that mediate photoallergy. It is possible that protein is covalently bound to a photodegradated site of phoptohapten to form an allergic photohapten–protein complex. Lysine may be a preferential amino acid to afford the binding site to a photohapten [45].

Chemical mechanisms for skin sensitization

Although the terms structure–activity relationship (SAR) and quantitative structure–activity relationship (QSAR) are widely used, for skin sensitization it is more meaningful to describe the relationship as being between chemistry and activity. Similarity in physicochemical properties rather than similarity in structure is a better indicator of whether two compounds will be similarly potent as sensitizers. Of course the structure determines the physicochemical properties, but not usually in a simple and transparent way.

Skin sensitization induction is a multi-stage process. Whether or not, and to what degree, exposure to a particular compound will result in skin sensitization, is dependent on events, or lack of events, at these stages [46]. In a recent analysis of published experimental evidence, the dependence of each stage on the structure of the sensitizer has been analyzed as follows [47].

1. Translocation of the sensitizer from the skin surface to the epidermal site of action. This depends on the dose given and duration of exposure, but is not strongly dependent on the chemical nature of the sensitizer.
2. Covalent reaction of the sensitizer with skin protein. This is strongly dependent on the chemical nature of the sensitizer, in particular electrophilic reactivity and hydrophobicity. The nature of the skin protein involved in this process is not established: possibilities range from any protein encountered in the skin to highly nucleophilic proteins, 'purpose-designed' by evolution, associated with epidermal Langerhans cell membranes.
3. Response of epidermal Langerhans cells to modified protein resulting in migration to the lymph node and presentation of antigen, leading to antigen recognition by naïve T-lymphocytes resulting in clonal expansion. These processes are described in more detail earlier in this chapter. They are not strongly dependent on the nature of the sensitizer, except indirectly in that they depend on the extent of protein modification produced in stage 2.

It follows that to induce sensitization a compound must be able, either directly or after metabolic or abiotic activation, to react covalently with skin protein. Early evidence for this fundamental chemistry-activity relationship was reported in 1936 by Landsteiner and Jacobs [48]. They investigated 20 benzene derivatives, variously substituted with halogeno and/or nitro groups. Ten of these compounds were found to be reactive towards methanolic aniline, used as a simple model for nucleophilic groups on proteins, and 10 were found to be unreactive. In guinea pig tests, all 10 aniline-reactive compounds (now recognized as S_NAr electrophiles, a term which was not current at that time) were found to be sensitizers, and all 10 non-reactive compounds failed to sensitize. Since this pioneering work, many other groups of compounds have been studied, with results supporting the fundamental chemistry–activity relationship [49–52]. A quantitative basis for this relationship is provided by the relative alkylation index (RAI) approach, on the basis of a mathematical model for covalent reaction (alkylation) of skin protein as a function of reactivity (expressed as a rate constant for reaction with a model nucleophile), hydrophobicity (expressed as a partition coefficient) and dose [53].

Attempts to develop 'global' quantitative structure-activity relationships (QSARs), covering a wide diversity of sensitizers, either by RAI-related approaches or by statistical approaches have not met with sufficient success to be predictively useful [54,55]. A more promising approach is to develop separate quantitative mechanistic models (QMMs), each based on reactivity and hydrophobicity parameters, for the major reaction mechanisms by which compounds can react with nucleophiles [56]. One of the reasons why this separation into domains is necessary is that the proportionality between reactivity with the skin protein and reactivity with a model nucleophile (or reactivity represented by computational

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Table 1. Examples for cutaneous ADRs (in alphabetical order) associated with various drug classes

Antibiotics (Betalactams)	Anticonvulsives	Antiretrovirals	NSAIDs
Acute generalized exanthematic pustulosis	Eczema-like eruption	Acne	Angioedema
Anaphylaxis	Exanthema	Lipodystrophy	Contact dermatitis
Drug induced pemphigus	Fixed drug eruption	Pigmentation	Exanthema (lichenoid)
Exanthema (morbilliform)	Gingival hyperplasia	Pruritus	Exanthema (morbilliform)
Exfoliative dermatitis	Hypersensitivity syndromes (DRESS)	Urticaria	Fixed drug eruption
Fixed drug eruption	SJS/TEN	Vasculitis	Photosensitivity
Pruritus	Vasculitis		Pruritus
Serum sickness-like reaction			Pseudoporphyria
SJS/TEN			SJS/TEN
Urticaria			Urticaria

indices or substituent constants) differs according to the reaction mechanism.

The major reaction mechanistic domains important in skin sensitization are: Michael acceptors (many botanical sensitizers belong in this domain), S_N2 electrophiles, Schiff base electrophiles, S_NAr electrophiles and acyl transfer agents. In many cases compounds can be confidently assigned to one of these domains by inspection of structure, using the guidelines shown in Table 1 [57]. In other cases assignment to a reaction mechanistic domain is not obvious, and requires chemical experimentation to determine how the compound reacts. An example is MCI 5-chloro-2-methyl-isothiazol- 3-one (MCI) and 2-methylisothiazol-3-one (MI) which, as a mixture of the two, are used as preservatives. From their structures, several possible reaction pathways could be proposed, but experiments with model nucleophiles revealed that S_N2 attack at sulphur is the key reaction, and in the case of MCI, which is the stronger sensitizer, can react further to give a highly electrophilic thioacyl chloride [58].

Some skin sensitizers are not directly protein-reactive, but are converted *in cutaneo*, either metabolically or abiotically, into electrophilic species. A well known example is the urushiols of poison ivy and related species: these are mixtures of 3-alkyl and 3-alkenyl catechols, and are thought to sensitize via oxidation to the corresponding *ortho*-quinones [49] (Michael acceptor domain) as shown in Fig. 3. Identifying features for sensitizers of this type, often referred to as pro-electrophiles or pro-haptens, have been listed in a recent review by Karlberg *et al.* [59].

Some chemicals are not skin sensitizers, but can undergo deterioration under certain storage conditions (e.g. prolonged exposure to air), giving rise to sensitizing impurities (e.g. autoxidation products). Such compounds are sometimes referred to as pre-haptens [60]. A distinction can be made between pro-haptens and pre-haptens. The latter undergo conversion to the 'ultimate sensitizer', to a sufficient extent to sensitize', in the timescale of exposure to the skin, and will consequently sensitize even if highly purified, whereas the former require longer timescales to generate the 'ultimate sensitizer' and will not sensitize when the skin is exposed to them as pure chemicals.

Although the large majority of sensitizers can be assigned to one of the five reaction mechanistic domains of Table 1, other mechanisms of protein binding sometimes need to be invoked. Some important examples are:

Allylic hydroperoxides (mostly tertiary, but some secondary) with the sub-structure C=C–C–OOH have been identified as skin-sensitizing autoxidation products of a variety of pre-haptens [58], such as limonene [61] (Fig. 4). They do not fit any of the domains in Table 1, and it has been suggested that they might react with proteins by a free radical mechanism involving homolytic cleavage of the O–O bond. Another possibility is that they could act as S_N1 electrophiles, dissociating by heterolytic cleavage of the C–O bond to give a resonance stabilized and selectively reactive carbonium ion C=C–C+.

Photosensitizers

These are compounds which can induce skin sensitization and elicit the sensitization response, but only when the skin is exposed to light. Photosensitizers absorb wavelengths of light present in sunlight, as a result of which a chemical species capable of binding to skin proteins is formed. Structural alerts for compounds able to act as sensitizers have recently been discussed in some detail [62].

Stimulated by developments such as the REACH legislation in the EU, there is currently a strong incentive to develop non-animal based approaches to assess skin sensitization potential of chemicals. Expert systems such as Derek for Windows [63] and TIMES-SS [64] based on the chemical mechanistic principles outlined above have an important role to play, in combination with a strategy along the lines [65].

Presented with a new compound

1. The first step is to classify it into its reaction mechanistic domain. One domain is the 'unreactive' domain, populated by predicted non-sensitizers. In some cases, experimental work will be needed to determine the reaction

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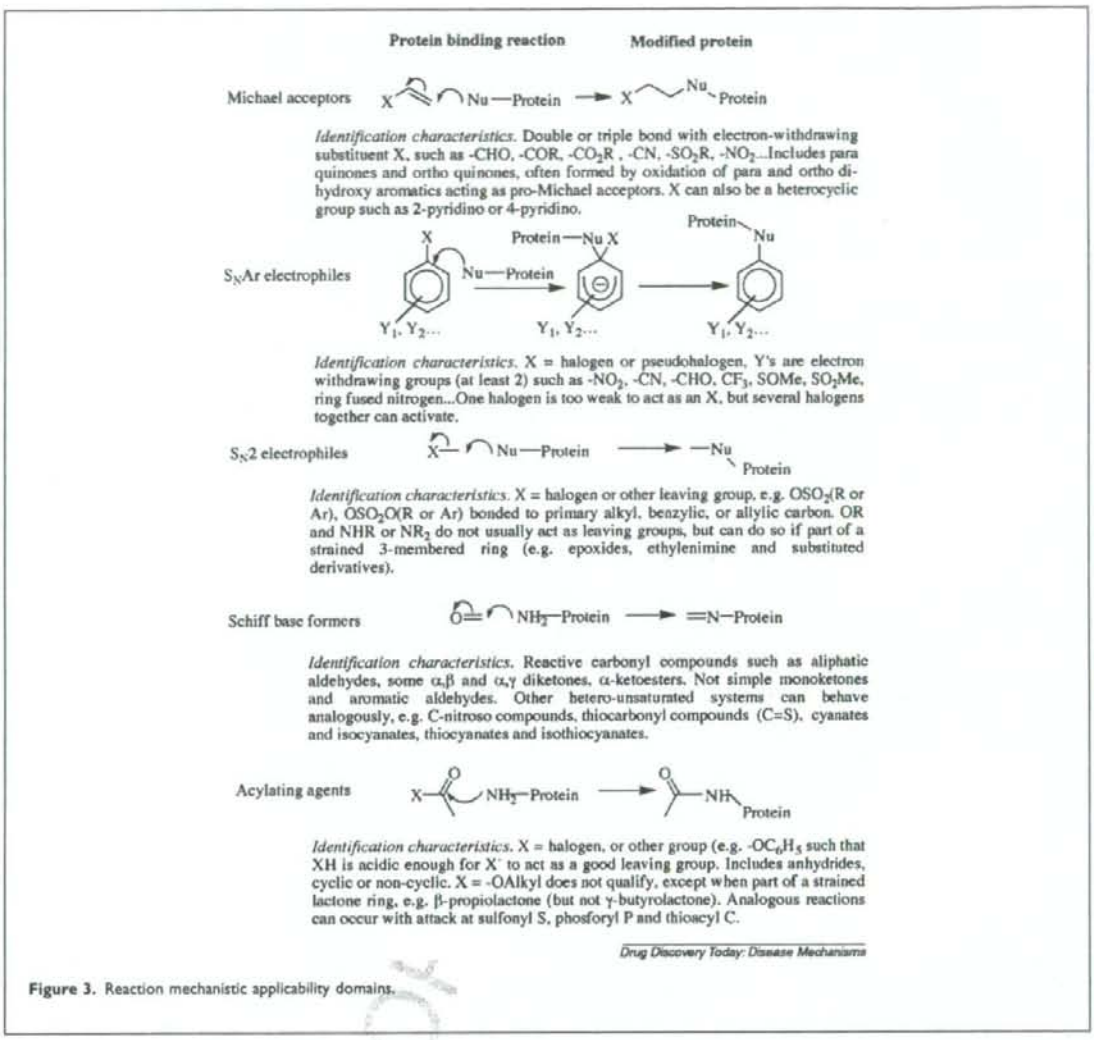

Figure 3. Reaction mechanistic applicability domains.

chemistry, in particular to determine if the compound is electrophilic or pro-electrophilic and the nature of the reactions.

2. The next step is to quantify its reactivity/hydrophobicity relative to known sensitizers in the same mechanistic applicability domain, either by calculation (e.g. on the basis of substituent constants or on molecular orbital parameters) or from physical organic chemistry measurements, such as reaction kinetics.
3. Having assigned the compound to its reaction mechanistic applicability domain and quantified its reactivity/hydrophobicity relative to known sensitizers in the same domain, QMM or mechanistic read-across [65] can be used to predict the sensitization potential.

Effect of topical products on the skin

Regulators classify chemicals as being hazardous after an acute dermal contact if they lead to mortality (owing to absorption), cause acute irritation or corrosion and cause skin sensitization on multiple contacts [66]. In the EU, evaluation of skin irritation/corrosion is mandatory for all products likely to be associated with skin exposure [66] (Fig. 1). Here, we examine direct irritation or corrosion effects. In doing so, we also recognize that chemicals applied to the skin can also have direct effects on the outermost barrier of the skin, the stratum corneum leading to an alteration in its permeability. The actions of these chemical penetration enhancers are discussed in more detail elsewhere [67]. Generally, three groups of enhancers exist – polar, semipolar and

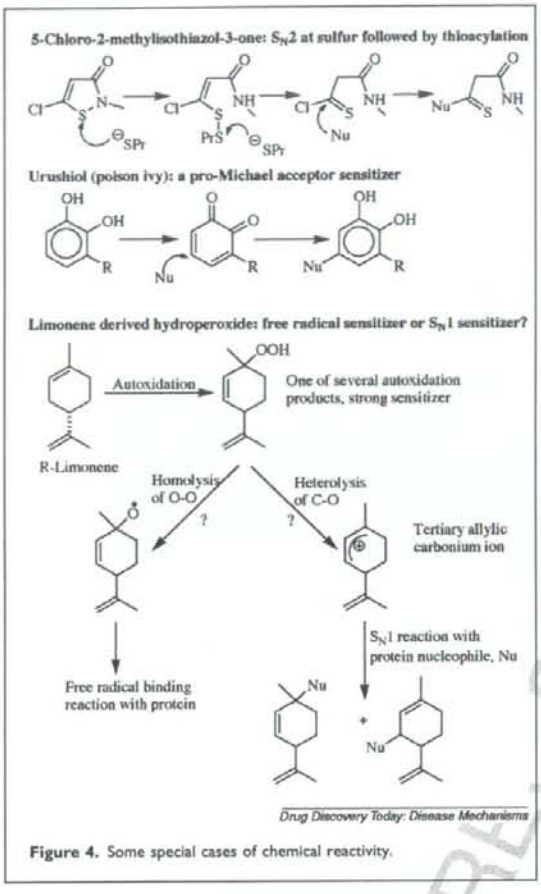

Figure 4. Some special cases of chemical reactivity.

lipid and each are generally small and, frequently, exist as liquids at low temperatures [67]. Polar penetration enhancers modify the polar region in intercellular lipids, in the corneodesomosomes (proteinaceous structures that hold corneocytes together) and possibly inside the corneocytes [67]. Water may be seen as a polar penetration enhancer in that it may increase skin permeability by up to 100-fold [68]. Other polar enhancers include urea, propylene glycol and glycerol. Moderately polar solvents also have the capacity to extract intercellular lipids and promote skin penetration, the effect being most pronounced when the solvent also undergoes strong hydrogen bonding, for example dimethyl sulphoxide and dimethyl formamide. Other ampiphilic solutes such as the surfactants can also promote penetration through the stratum corenum [67]. The lipophilic solvents and solutes can also extract lipids and their effects are most pronounced in changing the order of the lipophilic regions in the intercellular region of the stratum corneum. Examples here include the terpenes, hydrocarbons and certain oils [67].

A more severe form of penetration enhancement is that of corrosion in that the corrosive effects of the chemical tends not to be limited to the stratum corneum. Irreversible skin damage leading to visible necrosis of the epidermis through to the dermis after a 4 h application is defined as corrosion [66]. Dermal irritation, on the other hand, is generally reversible but is associated with either persistent erythema and scaling or, in the absence of erythema, severe oedema over a week [66] after an application for 4 h. As discussed elsewhere in this volume, corrosion and other directly acting chemicals may have a sequelae of irritant contact dermatitis [69]. Compounds associated with each of these processes work by the following mechanisms [66,70].

a. *Skin irritation*
   - Reaction with skin proteins and lipids by surface active agents.
   - Dissolution of skin lipids by low molecular weight organic chemicals.

b. *Skin corrosion*
   - Erosion of the stratum corneum by inorganic acids and bases and by strong organic acids with pH <2 and bases with pH >11.5.
   - Binding by cationic substances including surfactants, quaternary ammonium salts and sulphonium ions.

c. *Skin irritation and corrosion*
   - Penetration of the stratum corenum by certain chemical with ampiphilic properties.
   - Elicitation of a cytotoxic response in the epidermis or dermis.

The potential for skin irritation/corrosion is high if MW of chemical <250 g/mol, low if MW >370 g/mol and negligible if MW >1200 g/mol [70]. Compounds causing skin corrosion are generally of intermediate polarity (log octanol–water partition coefficients) of >−1 to <+6, are water soluble and generally melt below 40°C [70]. Our own experience with the phenolic and related compounds is that the formulation and solubility of the phenol may be a key predictor. For instance, phenol and the cresols have sufficient water solubility to reach a threshold concentration to cause irreversible skin damage (and corrosion) when given in water but require much higher concentrations in semipolar solvents owing to their reduction in phenol's chemical activity [71]. On the other hand, other more lipophilic and higher melting point phenols such as chlorocresol and chloroxylenol can be placed on the skin in a pure form without any apparent corrosion [71]. Skin corrosion also appears to be exclusive to the lower, as distinct from the higher, molecular weight fatty acids.

In general, corrosion and direct irritation differs from sensitization and allergic reactivity in that the former generally requires penetration through the stratum corneum as a

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prelude to triggering a response in the deeper epidermal or dermal layers. By contrast, the latter usually involves some covalent reaction with skin protein that, in turn, elicits an antigenic response with epidermal Langerhan cell mobilization.

Conclusions

Compounds applied to the skin have the potential to cause enhancement of skin penetration, corrosion, irritation and sensitization, with a resulting allergic or phototoxic response. In general, each of these processes requires groups of compounds to have particular chemical properties that enable the likelihood of such an effect to be predicted. Rules are now available that define which chemicals are most likely to be associated with each process and there is an increasing use of (Q) SARs and structural alerts to avoid undesirable compounds. As an example, care should be taken in designing topical products with the following groups: (hydro)peroxides, unsaturated substituents, aldehydes, heterocyclic three rings, (meth)acrylates, quinones, ketones and hydrazines [70].

There has also been an increasing motivation to use structure activity relationships to predict skin toxicity in relation to not only local but also skin manifestations after systemic drug administration. Toxicity and adverse effects of drugs is a major cause for new chemical entity failure to reach the marketplace. It is hoped that the increased understanding in skin reactivity to chemicals, which are being increasingly built on mechanistic foundations [1], will assist in predicting 'safe' new compounds.

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Expression of toll-like receptor 2, NOD2 and dectin-1 and stimulatory effects of their ligands and histamine in normal human keratinocytes

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None declared.

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Summary

Background Epidermal keratinocytes are involved in the skin innate immunity and express toll-like receptors (TLRs) and other innate immune proteins. The epidermis is continuously exposed to pathogenic Gram-positive bacteria or fungi. However, few studies have examined the function and expression of innate immune proteins in keratinocytes. Histamine, which is well known for itch and allergy, is closely associated with innate immunity, but its influence on epidermal innate immunity is still unclear.

Objectives To clarify the expression of innate immune proteins in keratinocytes stimulated by ligand pathogen-associated molecules, and the function of histamine in this process.

Methods We investigated the effects of lipopeptide (MALP-2, 1–100 ng mL^{-1}; ligand for TLR2), peptidoglycan (PGN, 0·02–2 μg mL^{-1}; ligand for NOD2) and β-glucan (1–100 μg mL^{-1}; ligand for dectin-1) in the presence or absence of histamine on mRNA expression of TLR2, NOD2 and dectin-1 as well as human β-defensin 2 by quantitative real-time polymerase chain reaction in cultured normal human epidermal keratinocytes. TLR2 expression was also examined at the cell surface and intracellularly, as determined by flow cytometry and confocal microscopy. The quantities of interleukin (IL)-1α and IL-8 produced by keratinocytes were measured using enzyme-linked immunosorbent assay.

Results At the mRNA level, TLR2 was enhanced by PGN but not by its ligand MALP-2 or by β-glucan; NOD2 was easily induced by all three ligands; and dectin-1 was enhanced by its ligand β-glucan. These enhanced expressions were further augmented by histamine at 1 μg mL^{-1}. While the surface expression of TLR2 was barely detectable by flow cytometry even after stimulation, the intracellular expression of TLR2 was apparently elevated by PGN and further promoted by histamine. A confocal microscopic analysis also revealed the enhanced expression of TLR2 in the cytoplasm. The expression of TLR2, NOD2 and dectin-1 was functional, as these pathogen-associated molecules induced the production of IL-1α, IL-8 and defensin, and again, histamine greatly enhanced this production.

Conclusions Our study demonstrated that the expression of functional innate immune receptors is augmented by the pathogen-associated molecules in a ligand-feed forward or nonrelated manner in keratinocytes, and histamine promotes their expression and the resultant production of cytokines and defensins.

Toll-like receptors (TLRs) are involved in the innate immune system and recognize various pathogen-associated molecular patterns of microorganisms, such as lipopeptide, lipopolysaccharide, RNA and unmethylated CpG DNA.[1] It has been shown by many studies that epithelial cells from several defensive organs play a pivotal role in the primitive defence system against microorganisms such as bacteria, fungi and viruses, and accordingly, they express TLRs.[2–4] TLR2 is a key

receptor for epithelial cells, because the epithelium is continuously exposed to pathogenic Gram-positive bacteria whose products stimulate TLR2. The expression of TLRs is induced by cytokines such as tumour necrosis factor (TNF)-α and interferon-γ,[4,5] and by TLR agonists per se.[6] In addition, it has been strongly suggested that mast cells are deeply involved in innate immunity[7] and in the epithelial defence system.[8,9] A recent finding that histamine, a key product from mast cells, induces TLR expression on endothelial cells,[10] implicates the ability of histamine to increase TLR expression in epithelial cells as well.

In the skin, keratinocytes are the first responders to external invaders and serve as initiators in innate immunity by producing cytokines/chemokines and antimicrobial peptides.[11–13] Several studies have documented that keratinocytes express TLRs such as TLR1, TLR2, TLR3, TLR4, TLR5, TLR6 and TLR10.[14] However, the induction and detection of these molecules remain unclear or even controversial among the previous reports. As assessed by flow cytometry, human keratinocytes were shown to express TLR4 as well as CD14,[15] whereas another group of investigators reported that only TLR2 was detected on the surface of cultured normal human keratinocytes.[16] The HaCaT keratinocyte cell line was reported to express both TLR2 and TLR4 by flow cytometry.[17] On the other hand, an immunohistochemical study of human skin demonstrated that TLR1, TLR2 and TLR5 are constitutively expressed in the cytoplasm of normal keratinocytes and that TLR2 expression is increased in psoriasis.[18]

Upon stimulation of keratinocytes via TLRs, they produce cytokines/chemokines and antimicrobial peptides as an outcome of operation of the innate immunity. These substances include interleukin (IL)-1α, TNF-α, granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-8, macrophage inflammatory protein-1α,[13,16,19–23] and major antimicrobial peptides, the β-defensins.[24–26] The production of these molecules is one of the hallmarks of expression of functional TLRs by keratinocytes. As a consequence, IL-1α, TNF-α and GM-CSF produced by keratinocytes subsequently activate the cutaneous acquired immunity by enhancing the antigen-presenting ability of dendritic cells.[13]

In this study, we aimed firstly to clarify the agents that induce the expression of TLR2 and its cooperating receptors in keratinocytes, focusing on lipopeptide, peptidoglycan (PGN), the fungal element β-glucan, and histamine. Secondly, we explored whether TLR2 expression can be assessed by flow cytometry at the surface or intracellular level. We chose these agents because lipopeptide is a TLR2 ligand[27] and β-D-glucan binds to dectin-1.[28] PGN, which is recognized by NOD2,[29,30] also augments expression of TLR2.[27,31] In addition to these external stimulants for epidermal keratinocytes, we also examined histamine, which is a physiological stimulator for TLR2 expression,[10] and whose receptors, H_1 and H_2,[32] are expressed on keratinocytes. Results suggest that PGN and histamine strongly elevate the intracellularly detectable TLR2 and augment the production/expression of cytokines/chemokines and β-defensin in normal human keratinocytes. It is notable that there is a synergism between the pathogen-associated molecules and histamine in the expression of TLR2 and the production of inflammatory and antimicrobial molecules.

Materials and methods

Culture and stimulation of keratinocytes

Normal human epidermal keratinocytes (NHEK) isolated from neonatal foreskin were obtained from Cambrex Bio Science Walkersville (Walkersville, MD, U.S.A.) and grown in the serum-free keratinocyte growth medium KGM-2 (Clonetics, San Diego, CA, U.S.A.) or Dulbecco's modified Eagle's medium (Gibco BRL Life Technology Inc., Gaithersburg, MD, U.S.A.) at 37 C in a 5% CO_2 incubator. NHEK were subcultured using trypsin–ethylenediamine tetraacetic acid (Clonetics), and semiconfluent cells at third passage were used in all experiments. Unless otherwise mentioned, semiconfluent keratinocytes in six-well plates (Corning Glass Works, Corning, NY, U.S.A.) with 2 mL of medium were stimulated with the following substances: lipopeptide (MALP-2; Axora, San Diego, CA, U.S.A.), PGN (InvivoGen, San Diego, CA, U.S.A.), β-glucan (MP Biomedicals, Aurora, OH, U.S.A.) and histamine (MP Biomedicals).

Quantitative real-time polymerase chain reaction

Total RNA from NHEK was isolated using the SV Total RNA Isolation System (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions with inclusion of the DNase step. Purified RNA was reverse transcribed with the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, U.S.A.) with oligo d(T)16 primers. TaqMan experiments were carried out in an ABI PRISM 7000 Sequence Detector System using TaqMan Gene Expression Assays for TLR1, TLR2, TLR6, NOD2, human β-defensin 2 (hBD2) and dectin-1 (Applied Biosystems). Endogenous β-actin was used to normalize the gene expression between different samples.

Flow cytometry of toll-like receptor 2 expression

NHEK were incubated for 48 h with the stimulants. For the surface expression of TLR2, cells were stained with phycoerythrin (PE)-conjugated anti-TLR2 (TL2.1) monoclonal antibody (mAb) or isotype control mouse IgG2a (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). For the intracellular expression of TLR2, cells were first stained with nonconjugated anti-TLR2 mAb, fixed with Cytofix/Cytoperm (BD Pharmingen, San Jose, CA, U.S.A.) for 40 min at 4 C, and stained with the PE-conjugated anti-TLR2 mAb as above. Hanks' balanced salt solution containing 0·1% NaN_3 and 1% fetal calf serum was used as the staining buffer. After incubation for 60 min at room temperature, cells were washed twice and analysed: fluorescent profiles were generated using a FACSCanto (Becton Dickinson, San Jose, CA, U.S.A.).

Quantification of cytokines and chemokines in keratinocyte culture supernatants

Three-day culture supernatants from NHEK were collected, stored at)80 C, and assayed for IL-1 α and IL-8 using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's directions. Optical density was measured with a microplate reader (model 3550; Bio-Rad, Hercules, CA, U.S.A.).

Construct and transfection

The pCMV-SPORT6-TLR2 (IMAGE clone 5213439) including full-length TLR2 cDNA (GenBank BC033756) and pCMV-SPORT6 (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) were purchased from Open Biosystems (Huntsville, AL, U.S.A.) and used for the transfection study. NHEK ($5 \cdot 10^5$ cells) were cultured without serum and antibiotics in a 60 - 15 mm TC dish (Nalge Nunc International, Naperville, IL, U.S.A.) and used at 40–50% confluency. Transfection was performed with 4 μg of plasmid DNA and 8 μL of Hily Max (Dojindo Laboratories, Kumamoto, Japan) in Opti-MEM I (Gibco). Four hours after transfection, the cells were washed and soaked in the fresh medium.

Toll-like receptor (TLR) 2 blocking with anti-TLR2 antibody in interleukin-8 production

Semiconfluent NHEK were incubated with 10 μg $mL^{)1}$ of Function Grade (FG) antihuman TLR2, clone TL2.1, or FG mouse IgG2a isotype control (eBioscience, San Diego, CA, U.S.A.) at room temperature for 1 h. PGN (0·02 μg $mL^{)1}$) or MALP-2 (1 ng $mL^{)1}$) was then added to the culture and incubated for 72 h. The concentration of IL-8 in the supernatants was measured by ELISA.

Immunocytoplasmic staining

NHEK were cultured in chamber slides (Nalge Nunc International). Slides were fixed and stained as for flow cytometric analysis. After staining, cells were analysed by confocal microscopy (LSM5 Pascal; Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Statistical differences were determined by Student's t-test or Welch's t-test; $P < 0.05$ was considered to be significant.

Results

Expression of mRNA for toll-like receptor (TLR) 2, its cooperating receptors TLR1 and TLR6, dectin-1, NOD2 and human b-defensin 2 in normal human epidermal keratinocytes

We first examined whether TLR2, its cooperating receptors (TLR1 and TLR6), dectin-1, NOD2 and hBD2 are expressed in unstimulated or stimulated NHEK. TLR2 is a receptor for lipopeptide (MALP-2), and dectin-1 in combination with TLR2 is a receptor for zymosan, and they collaborate with each other in recognition of microbes and induction of inflammation.[33–35] NOD2 is an intracytoplasmic molecule that recognizes PGN.[29,30] hBD2 is an antimicrobial peptide known to be produced following TLR ligation.[36,37]

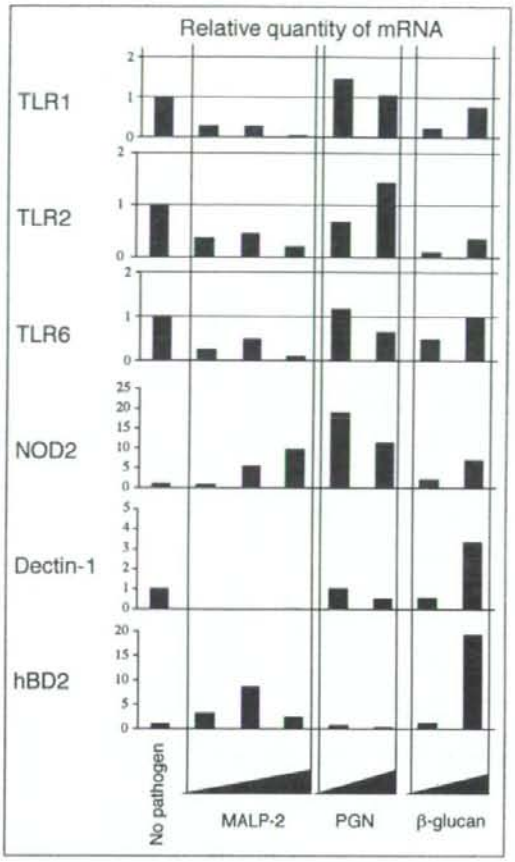

Fig 1. Expression of toll-like receptor (TLR) 2, its cooperating receptors TLR1 and TLR6, NOD2, dectin-1 and human β-defensin 2 (hBD2) in normal human epidermal keratinocytes (NHEK). NHEK were cultured for 2 h with MALP-2 (1, 10, 100 ng $mL^{)1}$), peptidoglycan (PGN) (0·2, 2 μg $mL^{)1}$) or β-glucan (1, 10 μg $mL^{)1}$) and subjected to real-time polymerase chain reaction analysis.

The levels of mRNA for TLR2, TLR1 and TLR6 were augmented by PGN to some extent at certain concentrations, but not by MALP-2 or β-glucan (Fig. 1). NOD2 expression was remarkably enhanced by all the stimulants, with variations. The expression of dectin-1 was elevated by β-glucan but not by MALP-2 or PGN. hBD2 expression was increased by MALP-2 and β-glucan. It is thus suggested that (i) NOD2 is easily inducible by various pathogenic stimulants, (ii) TLR2 is enhanced by PGN but not by its ligand lipoprotein or by

β-glucan; and (iii) dectin-1 is enhanced by β-glucan. These findings partly support the concept that TLR expression is often augmented by pathogen-associated molecules other than the corresponding specific ligand.[5,6]

Detection of augmented expression of intracellular toll-like receptor 2 by flow cytometry

We tested whether TLR2 is detectable at the protein level in NHEK, either unstimulated or following stimulation with PGN and β-glucan at relatively high doses. A flow cytometric analysis showed that the surface expression of TLR2 was very low, and that it was not enhanced by PGN (Fig. 2a) or β-glucan stimulation at either low or high Ca concentration, which induces basal and cornified keratinocytes, respectively. However, when these cells were subjected to intracellular staining, we found that PGN upregulated the expression of TLR2 (Fig. 2b). The addition of β-glucan also elevated TLR2 expression in the cytoplasm but not at the surface. Thus, an increased level of TLR2 was detected by flow cytometry in the cytoplasm but not at the surface of keratinocytes.

Functional expression of toll-like receptor 2, NOD2 and dectin-1 assessed by cytokine/chemokine production

To confirm the functional expression of TLR2, NOD2 and dectin-1, NHEK were cultured with MALP-2, PGN or β-glucan and the amounts of IL-1α and IL-8 secreted in the supernatants were measured. As shown in Figure 3, MALP-2 stimulated NHEK to produce these cytokines/chemokines. The NOD2 ligand PGN also augmented the production at an optimal concentration as low as 0·2 μg mL^{-1}. Similarly, β-glucan, a ligand for TLR2 and dectin-1, promoted the production of these cytokines/chemokines at an optimal concentration of 10 μg mL^{-1}. Thus, TLR2 and NOD2 seemed to be functionally expressed in NHEK.

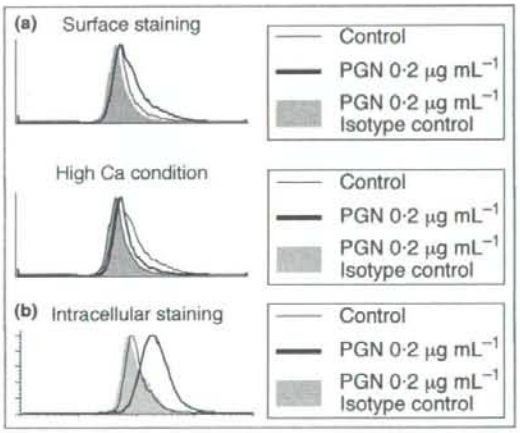

Fig 2. Flow cytometric detection of toll-like receptor (TLR) 2 expression in the cytoplasm but not on the surface of normal human epidermal keratinocytes (NHEK). NHEK were cultured in the presence or absence of peptidoglycan (PGN) for 48 h. Cells were stained with anti-TLR2 monoclonal antibody (mAb) or isotype-matched control antibody (a). The levels of isotype-matched control were evaluated in the stimulated NHEK. Simultaneously, NHEK cultured with the stimulant were intracellularly stained with anti-TLR2 mAb (b).

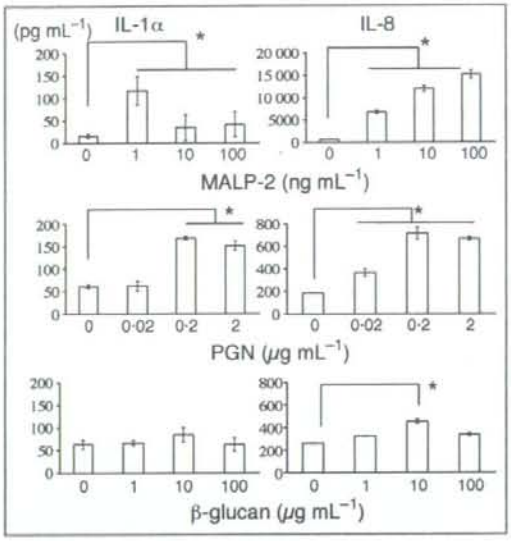

Fig 3. Augmentative effect of MALP-2, peptidoglycan (PGN) and β-glucan on cytokine/chemokine production by normal human epidermal keratinocytes (NHEK). NHEK were cultured for 72 h in the presence or absence of pathogen-associated molecules at the indicated concentration. The amounts of interleukin (IL)-1α and IL-8 in the supernatants were measured by enzyme-linked immunosorbent assay. The values are means of quadruplicate determinations with SD shown by vertical bars. *P < 0·05, compared with the nonaddition group.

It is known that PGN is not a ligand for TLR2 but is an activator.[31,38,39] To confirm this notion, we performed two studies. In one study, TLR2-transfected keratinocytes were stimulated with PGN or MALP-2, and the concentration of IL-8 was measured in the culture supernatants. Compared with the control (mean ± SD 1970·45 ± 15·14 pg mL^{-1}), addition of MALP-2 at 1 ng mL^{-1} (25180·1 ± 26·34 pg mL^{-1}) but not PGN at 0·2 μg mL^{-1} (1957·83 ± 28·44 pg mL^{-1}) gave a significantly higher level of IL-8 secretion in the supernatants. In the other study, NHEK were cultured with PGN (0·2 μg mL^{-1}) or MALP-2 (1 ng mL^{-1}) in the presence of blocking antibody to TLR2 or IgG2a as isotype-matched control. Following stimulation with PGN, neither anti-TLR2 antibody-treated nor isotype-matched control showed decreased production of IL-8. Following stimulation with MALP-2, however, treatment with anti-TLR2 antibody, but not control IgG2a, significantly decreased IL-8 production by 20%. Taken together, these findings suggest that PGN is not a specific ligand for TLR2.

Synergistic effects between pathogen-associated molecules and histamine on toll-like receptor 2 expression and cytokine production

NHEK were cultured with PGN or β-glucan in the presence or absence of histamine, and the levels of mRNA expression for TLR1, TLR2 and TLR6, NOD2, dectin-1 and hBD2 were measured by quantitative real-time polymerase chain reaction (PCR). Histamine at 1 μg mL^{-1} clearly amplified the expression of all the receptors and hBD2 that were induced by MALP-2 at 10 ng mL^{-1}, PGN at 2 μg mL^{-1} or β-glucan at 10 μg mL^{-1} (Fig. 4), demonstrating their synergistic effects.

Further to confirm the synergism by intracellular flow cytometry, we tested the combined effect of PGN at 0·2 μg mL^{-1} and histamine at 1 μg mL^{-1} on the expression of TLR2. The synergism between PGN and histamine was clearly observed, as the addition of both further elevated the TLR2 expression induced by individual stimulants (Fig. 5a), but again the surface expression was undetectable (data not shown). A confocal image analysis of keratinocytes showed that augmentation of the cytoplasmic level of TLR2 induced by synergism between PGN and histamine was greater than that of the cell surface level (Fig. 5b).

Finally, the synergism with histamine in cytokine/chemokine production was examined. NHEK were cultured with MALP-2, PGN or β-glucan in the presence or absence of histamine, and the amounts of IL-1α and IL-8 in the supernatants were measured. As shown in Figure 6, histamine at 1 μg mL^{-1} alone did not increase the production of IL-1α or IL-8 (solid bars of ligand 0). However, histamine upmodulated IL-1α and IL-8 production in the presence of MALP-2, PGN or β-glucan.

Discussion

Our study demonstrated that NHEK express both TLRs and nontoll-like innate immune proteins, including the intracellular Nod-like protein NOD2 and the surface C-type lectin dectin-1. As MALP-2, PGN and β-glucan stimulated keratinocytes to produce cytokines and antimicrobial peptides, it is considered that TLR2, NOD2 and dectin-1 are functionally expressed and efficiently mount the innate immunity in keratinocytes. More interestingly, the innate immune receptors were augmented by pathogen-associated molecules relevant or irrelevant to the corresponding receptors, as has been suggested in other types of cells.[5,6] The feed-forward stimulation was observed between PGN and NOD2 and between β-glucan and dectin-1, while TLR2 was induced by PGN but not by its ligand MALP-2. Therefore, the pathogen-driven acceleration of receptor expression appears to be complicated. However, NOD2 might be a key molecule, because all the pathogen-associated molecules tested enhanced NOD2 expression, and its ligand PGN was most stimulatory for the cytokine/hBD2 production among the pathogen-associated molecules.

TLR2 was induced by PGN and was detected at both protein and mRNA levels. We successfully evaluated the intracellular,

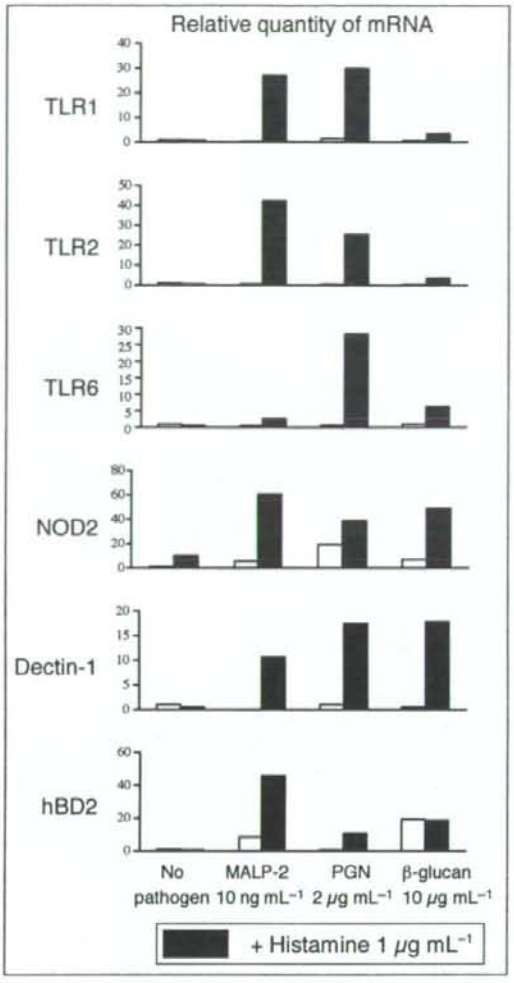

Fig 4. Synergism of MALP-2, peptidoglycan (PGN) or β-glucan, with or without histamine, in mRNA expression for toll-like receptor (TLR) 1, TLR2, TLR6, NOD2, dectin-1 and human β-defensin 2 (hBD2) by real-time polymerase chain reaction (PCR) analysis. Normal human epidermal keratinocytes were cultured for 2 h with each of the pathogen-associated molecules and/or histamine and subjected to real-time PCR analysis.

but not surface, expression of TLR2 by flow cytometry. The expression of each TLR in keratinocytes has been a matter of debate.[40] In the present study, the surface expression of TLR2 was very low compared with the isotype-matched control, and was not enhanced by any of the stimuli at either low or high Ca concentration. Nevertheless, we found that the intracellular expression of TLR2 was significantly elevated upon stimulation. As the TLR2 ligand MALP-2 promoted the production/expression of cytokines and of hBD2, it is considered that TLR2 is expressed on keratinocytes as a functional surface molecule. Although the change of surface expression was

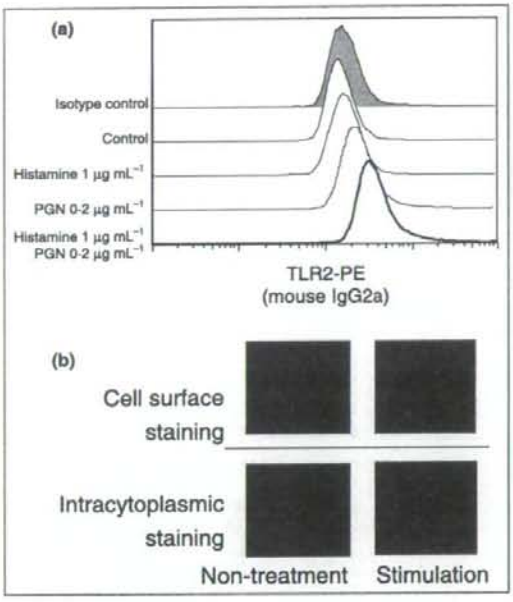

Fig 5. Synergism between peptidoglycan (PGN) and histamine in intracellular toll-like receptor (TLR) 2 expression and confocal microscopic analysis for detection of TLR2 in normal human epidermal keratinocytes (NHEK). (a) NHEK were cultured in the presence of histamine (1 μg mL^{-1}) and ⁄or PGN (0·2 μg mL^{-1}), and were subjected to intracellular staining for TLR2. The level in the isotype-matched control was evaluated in NHEK stimulated with both histamine and PGN. (b) NHEK were cultured in a chamber slide in the presence or absence of PGN at 0·2 μg mL^{-1} and histamine at 1 μg mL^{-1} for 48 h. After incubation with nonconjugated isotype control mouse IgG2a, intracellular or cell surface staining of keratinocytes was performed with phycoerythrin (PE)-conjugated anti-TLR2 monoclonal antibody and visualized by confocal microscopy.

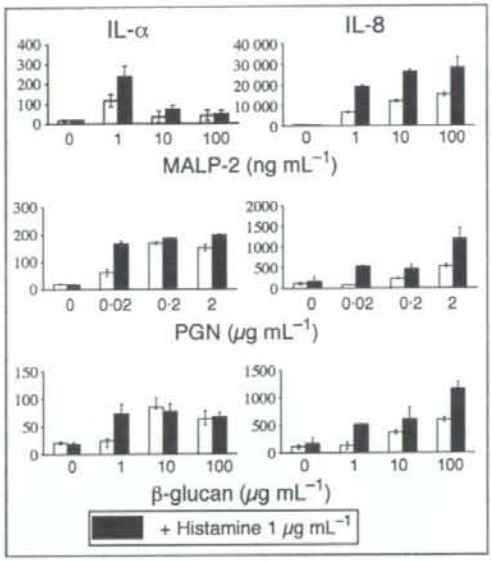

Fig 6. Synergism between pathogen-associated molecules and histamine in cytokine ⁄chemokine production by normal human epidermal keratinocytes (NHEK). NHEK were cultured for 72 h with each of the pathogen-associated molecules and ⁄or histamine. The concentrations of interleukin (IL)-α and IL-8 in the supernatants were measured by enzyme-linked immunosorbent assay. The values are means of quadruplicate determinations with SD shown by vertical bars. PGN, peptidoglycan.

under the detection level, the intensity of its intracellular expression may predict an alteration of the surface expression. A similar finding has been reported in dendritic cells[41] and has recently been reported in keratinocytes.[42] On the other hand, a recent finding has suggested that the intracellular TLR2 functions as a receptor for the infecting pathogen.[43] Considering that skin is constantly exposed to microorganisms, the low level of surface expression of TLR2 might be reasonable. If the quantity of TLR of the surface of keratinocytes were easily increased, this would always give rise to an irritable response to pathogens and to the occurrence of inflammation. In this respect, keratinocytes should be different from monocytes ⁄macrophages, and intracellular TLR2 appears to recognize the pathogens when they invade the cell. There might exist a certain protein that regulates the surface expression of TLR2 like that of TLR4,[44] and it may act on the homeostasis of the epithelial tissue.

It is already known that histamine plays an important role for innate immunity.[45] We stimulated keratinocytes with the pathogen-associated molecules in combination with histamine. Histamine amplified the expression of TLRs and proinflammatory cytokines synergistically with the pathogen-associated molecules. The augmentation of TLR2 expression by histamine was also observed in endothelial cells.[10] We found that there was synergism between histamine and MALP-2, PGN or β-glucan in TLR2 expression, as assessed by both intracellular staining and real-time PCR. Such synergistic effects were also found in the expression of hBD2 and dectin-1 and the production of IL-1α and IL-8. In the skin, keratinocytes are potentially exposed to histamine that is released from dermal mast cells in certain pathological conditions. As mast cells also express TLRs and can produce histamine by TLR ligation,[46] pathogens may stimulate keratinocytes directly or indirectly via mast cells with their produced histamine. This scenario suggests the potential involvement of histamine in the natural defence system and may result in an exaggerated response to pathogens.

Our findings are of clinical significance. As the skin is constantly exposed to Gram-positive bacteria and fungi as exemplified by Staphylococcus aureus colonization[47] and superficial fungal infection,[48] the upmodulation of TLR2, NOD2 and dectin-1 in keratinocytes may be beneficial for the defence system. The expression of these molecules is enhanced by pathogens, resulting in the augmented production of

proinflammatory cytokines and chemokines for neutrophils and lymphocytes. These cytokines/chemokines eventually protect the host from bacteria and fungi by inducing inflammation and immune reactions. In addition, the upregulated production of the defensins effectively eliminates microorganisms. Histamine may support these events as an internal stimulus for innate immune protein expression. It is suggested that the activation of keratinocytes via innate immune proteins leads to pluripotential responses in the cutaneous innate immunity and subsequent acquired immunity.[13]

Acknowledgments

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Table 1 *Ex vivo* skin penetration and permeation of eucalyptol (μg/cm²; mean ± S.D.; $n = 4$)

Amount of eucalyptol	Child skin			Adult skin		
	15 min	30 min	60 min	15 min	30 min	60 min
Stratum corneum total	712 ± 43	763 ± 40	698 ± 78	564 ± 46	549 ± 59	575 ± 127
Epidermis with *dermis*	460 ± 184	1248 ± 281	1357 ± 158	319 ± 120	543 ± 54	845 ± 98
Skin total	1172 ± 98	2011 ± 170	2055 ± 193	883 ± 138	1092 ± 108	1420 ± 161
Acceptor fluid	0	45 ± 16	73 ± 17	0	0	0

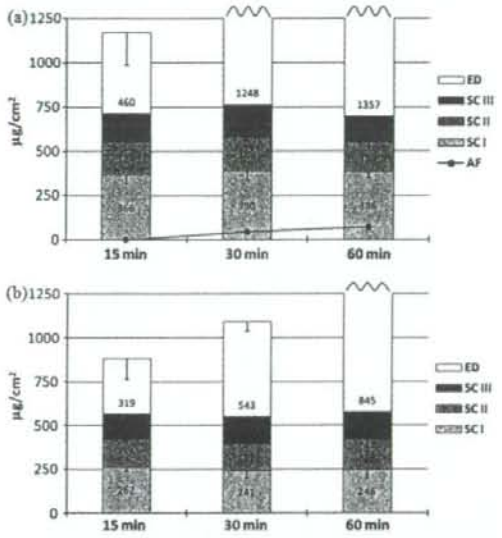

Fig. 1 Absorption of eucalyptol (mean ± S.D., $n = 4$). (a) In child skin layers. (b) In adult skin layers. *Abbreviations*: ED, *epidermis* with *dermis*; SC III, inner *stratum corneum*; SC II, middle *stratum corneum*; SC I, outer *stratum corneum*.

Even if side effects for eucalyptol are sporadically reported, the possibility of their appearance when using eucalyptol containing drug products should be appreciated. The molecular mass of eucalyptol (<500 Da) predicts it to have a possible allergenic factor [8]. This seems to be essential for the application of eucalyptol containing preparations onto the skin of young children because their skin can be outright "open" for exogenous substances.

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LETTER TO THE EDITOR

Evaluation of photoallergic potential of chemicals using THP-1 cells

KEYWORDS
Photoallergy; Photohapten; Prohapten; THP-1

Both systemic and topical exogenous photosensitizers evoke cutaneous photosensitivity and clinically recognized drug photosensitivity and photocontact dermatitis, respectively. The action spectrum of these two types of photosensitivity is mainly ultraviolet A (UVA) [1]. Photosensitive chemicals have both phototoxic and photoallergic potentials. Phototoxic reaction is mainly mediated by free