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CONTACT DERMATITIS

Review Article

Hapten–protein binding: from theory to practical application in the *in vitro* prediction of skin sensitization

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In view of the forthcoming European Union ban on *in vivo* testing of cosmetic and toiletry ingredients, following the publication of the 7th amendment to the Cosmetics Directive, the search for practical, alternative, non-animal approaches is gathering pace. For the end-point of skin sensitization, the ultimate goal, i.e. the development and validation of alternative *in vitro*/*in silico* assays by 2013, may be achieved through a better understanding of the skin sensitization process on the cellular and molecular levels. One of the key molecular events in skin sensitization is protein haptenation, i.e. the chemical modification of self-skin protein(s) thus forming macromolecular immunogens. This concept is widely accepted and in theory can be used to explain the sensitizing capacity of many known skin sensitizers. Thus, the principle of protein or peptide haptenation could be used in *in vitro* assays to predict the sensitization potential of a new chemical entity. In this review, we consider some of the theoretical aspects of protein haptenation, how mechanisms of protein haptenation can be investigated experimentally and how we can use such knowledge in the development of novel, alternative approaches for predicting skin sensitization potential in the future.

Key words: hapten–protein binding; *in vitro* assay; peptide binding; skin sensitization. © Blackwell Munksgaard, 2005.

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A skin sensitizer is a chemical with an intrinsic ability to induce contact allergy. Once sensitized to that chemical, the person is susceptible to elicitation of the symptoms of allergic contact dermatitis (ACD) upon subsequent exposure to the same or cross-reactive chemicals. ACD is a delayed (type IV) hypersensitivity reaction to an exogenous chemical mediated by T-cell-related processes (1–3). It is estimated that ACD affects a significant proportion of the general population (approximately 1%) (4, 5). In affected individuals, it has a serious impact on their quality of life. Contact allergens are present in the natural environment, but the potential exposure to allergens becomes a greater regulatory issue within industry when they could be present in consumer products, for example, personal care products that are intended for application to the skin. Industries assure the safety of all chemical ingredients in their products with respect to skin sensitization using state-of-the-art risk assessment tools.

Assessment of the potential skin sensitization hazard and relative potency of chemicals is currently reliant on *in vivo* methods such as the local lymph node assay (LLNA) (6), as no validated *in vitro* alternatives exist to date (7). Whilst computational sensitization hazard prediction rulebase tools such as Deduction and Estimation of Risk from Existing Knowledge (DEREK) may be useful to some extent in the initial screening of potential sensitization hazard of chemicals in early product development, these tools are based largely on the theoretical predictions of chemicals possessing the ability to react with proteins, and hence these methods do not estimate potency and are not used as risk assessment tools in safety support (8). A European Union ban on *in vivo* testing of cosmetic and toiletry ingredients will come into force in 2013 for the end-point of skin sensitization (9). Hence, there is an urgent need in the European cosmetics and toiletries industry to develop truly novel, alternative methods for both hazard identification and potential potency assessment of skin sensitizing chemicals in order that accurate risk assessments can continue to be derived for humans in the

absence of *in vivo* animal data. It is likely that improving our understanding of the cellular and molecular mechanisms of the sensitization process will result in novel opportunities for the development of alternative methods for assessing skin sensitization hazard and relative potency of chemicals.

On a cellular level, several approaches are currently being investigated to expand our currently limited understanding of the skin sensitization process. For example, the role of Langerhans' cells and regulation of the functional and phenotypic changes these cells undergo during sensitization are a subject of a number of investigations (10). Langerhans' cells are cutaneous, immature, dendritic cells (DCs) that recognize and internalize hapten–protein conjugates. Regulated by cutaneous cytokines, the mobilization towards the regional lymph nodes and concomitant maturation (to DCs) of these cells are induced during sensitization. At the same time, these cells process and present the antigen on their surface associated with the major histocompatibility complex class II (MHC II) molecules. This complex is subsequently recognized by naïve T cells, thus instigating clonal expansion of allergen-specific T lymphocytes and acquisition of cellular immunological memory. Casati et al. (10) have reviewed studies that have concentrated on identifying reliable cytokine or cell-surface biomarkers in chemically treated DCs, which could then in theory provide a read-out of a predictive test for sensitization potential of chemicals. Additionally, there are a number of other published cell culture studies where responses to treatment with sensitizers have been investigated, including keratinocyte cultures and DC : T-cell cocultures (11–14). Signal transduction pathways in DCs, such as mitogen-activated protein kinases and nuclear factor-κB pathways, have also been a subject of several investigations in search of reliable markers (15, 16). It will be important in such cell-based assays to introduce the test chemical into the system in the 'right' way to give good predictivity. For example, we do not know the metabolic competency of DCs, and the chemical may need to be metabolically preactivated or oxidized prior to addition in the test system, or the chemical may need to be presented to the DC as a protein–hapten or peptide–hapten complex, rather than just added to the system as chemical alone. These are questions that still need to be resolved, as we do not know all of the molecular mechanisms of DC stimulation.

On a molecular level, exploring the mechanisms of hapten–protein binding in relation to the early stages of the skin sensitization process should lead to useful insights for the development of *in vitro* assays (17, 18). The basis of hapten–protein binding work is the hypothesis that upon skin absorption, only protein-reactive chemicals (or those that can be metabolically or chemically converted to protein-reactive species) are able to act as skin sensitizers and that they do this through a process of protein haptenation (19, 20). Chemicals may potentially react with many different skin proteins at many different amino acid sites, but in general, protein molecules are rich in nucleophiles and the sensitizing chemicals are reactive electrophiles. One can theorize about the potential mechanisms that lead to skin sensitization (5), but very few of them have been proven experimentally, and when investigations are performed on specific chemicals, it is often the case that experiment shows much more mechanistic complexity than was originally theorized. Some investigators would refute the 'covalent binding' hypothesis of protein haptenation in favour of either non-covalent modes of protein–hapten association or indeed the modification of the normal self-protein-processing pathways, thus forming 'cryptic' epitopes that are recognized as foreign peptides (21). It is therefore important that we fully understand the links between protein haptenation and the ability of a chemical to cause sensitization. An increased understanding of protein haptenation mechanisms *in vitro* should also increase our confidence in hazard predictions *in silico*, which are based on the input of mechanistic knowledge, and could also lead to the development of simple, cost-effective and medium throughput *in vitro* protein or peptide haptenation assays for sensitization hazard and potency identification.

Chemistry

The concept

In 1935, Landsteiner and Jacobs published a landmark paper (20) in which they postulated that a small organic molecule can become a sensitizing entity only once it is bound to a skin protein. Their observations were based on studies of guinea-pig sensitization to 2,4-dinitro-1-chlorobenzene (DNCB). Sensitizing chemicals are too small to be recognized by the classical immunological mechanisms, and therefore, they need to be protein bound in order to elicit an immune response. Unless already a protein-reactive molecule, a sensitizer may be chemically or metabolically activated prior to or upon cutaneous absorption. Subsequently, it must bind to skin protein(s) to form a macromolecular immunogen.

Since those first attempts, a number of studies have been conducted on the subject of protein haptenation in an effort to detect, characterize and quantify the process. Although not always related to skin sensitization, these studies have nevertheless achieved important milestones in our understanding of protein haptenation mechanisms (22–29). Over recent years, methodology applied to characterize protein–hapten binding has improved substantially, including immunochemistry, nuclear magnetic resonance (NMR) and mass spectrometry. These improvements have now enabled us to investigate the specificity as well as the broad extent of protein binding using a variety of skin sensitizers. The theory postulated by Landsteiner and Jacobs remains to be proven in terms of skin sensitization, but its wide acceptance results from investigations of numerous examples of skin sensitizers and their ability to covalently modify proteins. Theoretical mechanisms of reactions can be postulated for the majority of direct acting sensitizers, and increasingly, mechanisms for prohaptens (those compounds that are not protein reactive *per se*) can also be explained mechanistically as our knowledge of skin metabolism increases (30–32).

The reactions of chemicals with proteins lead to the formation of bonds of different strengths. The formed chemical bonds are characterized by their energies, which are a direct reflection of their stabilities. Weak interactions, such as hydrophobic, dipolar (including hydrogen bonds) and ionic bonds, involve energies of up to 50 kJ/mol, whereas strong interactions, such as covalent and co-ordination bonds, involve energies ranging from 200 to 420 kJ/mol (33). The evidence to date indicates that the interaction between the hapten and protein must result in a formation of a strong bond so that a non-self antigen is produced.

Considering the chemicals

For protein haptenation to occur (and hence lead to skin sensitization), a chemical must be electron deficient (electrophilic). Chemicals are electrophilic if they either (i) have a polarized bond (such as halogenated compounds, aldehydes, ketones and amides), or (ii) are unsaturated compounds conjugated with electron-withdrawing groups (e.g. α,β-unsaturated aldehydes, nitrobenzenes, etc.) (Fig. 1) or (iii) are cations (Ni^{2+} and Cr^{3+}). As previously mentioned, a chemical may not be initially electrophilic but can be converted to a protein-reactive species by air oxidation or cutaneous metabolism. Examples of such reactions are shown in Fig. 2.

It is prudent to assess a broad range of chemicals during investigations of new techniques and assay development. A chemical dataset for evaluation of alternative approaches to skin sensitization testing was recently published (34). These recommended materials encompass chemical and biological diversity of known chemical allergens as well as providing suitable negative controls.

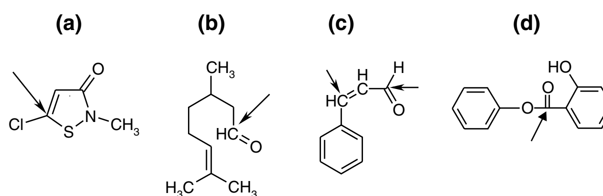

Fig. 1. Examples of structures of the electrophilic sensitizing xenobiotics (electrophilic centres indicated by arrows) (a) 5-chloro-2-methyl-4-isothiazolin-3-one (MCI), (b) citronellal, (c) cinnamaldehyde and (d) phenyl salicylate.

Considering the target

The main targets of small molecule electrophiles are amino acid side chains with nucleophilic properties. Nucleophiles either have atoms containing 1 or more unshared pairs of electrons or are negatively charged ions. The strongest potential nucleophiles in proteins, apart from the N-terminal amino group, are the lysine ϵ-amino group, the cysteine sulfhydryl group and the histidine imidazole group. The ability of amino acid side chains to react with electrophilic chemicals is largely dependent on the degree of ionization (Fig. 3), bearing in mind that the skin pH ranges from 5.5 on the surface of the stratum corneum to physiological 7.4 in the epidermis and dermis (5). The ability of a chemical to react with a nucleophile may be hindered or enhanced by the nucleophile position in a 3-dimensional protein environment. For example, a nucleophilic side chain may be embedded amongst residues with hydrophobic, non-polar side chains making it less accessible to hydrophilic chemicals

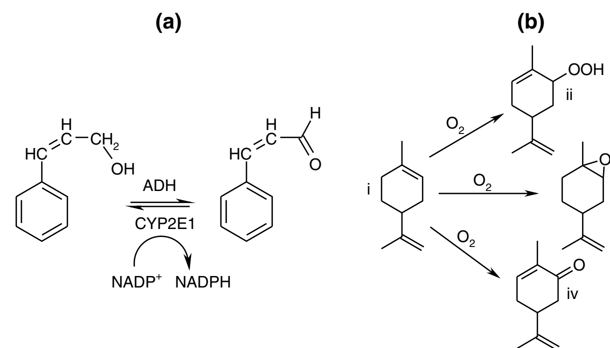

Fig. 2. (a) Metabolic conversion of cinnamic alcohol to cinnamaldehyde catalysed by cutaneous ADH or CYP2E1 with NADP+ as cofactor (36), (b) air oxidation products of (i) *d*-limonene, (ii) limonene-2-hydroperoxide, (iii) limonene epoxide and (iv) carvone (69).

	Protonated (pH < pKa)	Deprotonated (pH > pKa)
Carboxy terminus pKa = 2		
Amino terminus pKa = 10		
Asp (D) pKa = 4		
Glu (E) pKa = 4		
His (H) pKa = 6		
Lys (K) pKa = 10		
Arg (R) pKa = 12.5		
Cys (C) pKa = 8		
Ser (S) pKa = 13		
Thr (T) pKa = 13		
Tyr (Y) pKa = 10		

Fig. 3. Protonated and deprotonated forms of ionizable amino acid side chains and N- and C-terminus (R-amino acid side chain, X,X′-amino acid).

but more accessible to lipophilic compounds. Similarly, depending on the surrounding amino acids, the pH of the local microenvironment may be dramatically different from the pH of the surrounding medium, thus greatly influencing the degree of ionization of nucleophilic side chains. The degree of side chain ionization may also be altered depending on the site of haptenation *in vivo*, given that some intracellular compartments are more acidic than the cytoplasm or the extracellular matrix.

On a macromolecular level, although a lot is known about skin anatomy and physiology, the complete profile of proteinaceous constituents of the skin has not yet been fully established. Skin as a complex heterogenous tissue expresses a large number of proteins. Over 2000 proteins have been separated in cultured human keratinocytes, but approximately only a third of those could be identified using proteomic techniques (35). It is logical to assume that a protein-reactive chemical will modify any available nucleophile to some extent given suitable conditions of reactivity. A recent study investigating the binding of cinnamaldehyde to human skin homogenates showed that this moderate sensitizer was bound to a broad range of proteins in the sample, and there did not appear to be any specific targeting to any particular proteins (36). In the absence of a target skin protein *per se*, *in vitro* investigations have been limited to the use of model proteins or peptides to explore general chemistries.

Protein/peptide models for studying peptide haptenation

Human serum albumin (HSA) is often the model protein of choice for protein-binding assays. This

is a well-characterized protein, and around 40% of extravascular HSA is located in the skin (37). The role of albumin in blood and tissues *in vivo* is often to bind to xenobiotics via its substrate-binding pockets and remove the invading chemical from the circulation or tissue, thus acting as a detoxification mechanism. HSA has been used in numerous protein-binding studies to provide routine assessments of non-covalent plasma protein-binding levels for many drugs and pharmaceuticals, and such data are useful for pharmacokinetic analyses. In protein haptenation studies, HSA is used as a model protein tool to look at protein binding in a different way, i.e. the direct chemical modification of the protein residues via specific chemical reactions. Several key HSA residues have been shown to be selectively and covalently modified by chemicals, such as Cys 34 (38) Lys 190 (39), Lys 199 (28, 40, 41), His 9, His 146, His 338 (42) and Arg 410 (22). HSA has 17 pairs of cysteines involved in disulfide linkages and only 1 free Cys residue (Cys 34). Similarly, there is only 1 Trp residue on HSA. Therefore, it should be remembered that HSA is only one choice of macromolecular tool and has its own specific properties. Other macromolecules may show different mechanisms of protein haptenation for the same chemical, dependent upon their macromolecular properties. The question about the immunological relevance of specific residue modifications in macromolecules remains. For example, it has been suggested that a tolerizing effect may be due to exclusive modification of sulfhydryl groups (43). Hence, care should be taken in interpreting the relevance of protein haptenation studies against the existing knowledge of the *in vivo* skin sensitization properties of the chemical investigated.

Peptides with sequences analogous to a part of human proteins are also used to assess chemical reactivity. DS3 peptide, which has a sequence analogous to the N-terminal part of the human globin (sequence VLSPADKTNW GHEYRMFCQIG), was used to investigate binding of 4-chloro benzenediazonium hexafluorophosphate (27), acetaldehyde (24) and 5-chloro-2-methylisothiazol-3-one (44). Glycine apart, this peptide contains 1 residue of each of the commonly occurring amino acids. The Cys residue of the peptide is often carboxymethylated or simply omitted from synthesis to avoid peptide dimerization in reactivity studies. Similarly, the synthetic peptide PEPAKSAPAPKKGSKKAVTKAQK, which represents the N-terminal part of human histone H2B (residues 1–23), was used in the reaction of phosgene, the major active metabolite of chloroform (29).

There are also examples of binding studies where peptides used are unrelated to any protein. For example, the complex reactions of 2-alkenals with proteins were investigated using a short model peptide, N-acetylglycyllysine *O*-methyl ester (AcGKOMe) (23). Similarly, a synthetic peptide PHCKRM, which exclusively contains common nucleophilic amino acids, was used to investigate the binding of 1,4-benzoquinone (45), 4-*t*-butyl-1,2-benzoquinone (46) and 2 metabolites of a prohapten (5R)-5-isopropenyl-2-methyl-1-methylene-2-cyclohexene (45, 47).

The major drawback of using small peptides is the further removal from the biological environment a hapten might find itself exposed to once it penetrates the skin, as the potentially crucial influence of 3-dimensional protein environment is not represented. However, model peptides have their place in such investigations, in proving chemistries that may currently only be theoretical for many sensitizers and showing new potential chemistries (5, 19). A further advantage is the simpler analytical process for short peptides and thus the potential for a medium or high throughput assay to be developed. It is also technically easier to investigate the relationship between the extent of the binding of chemicals to certain amino acid residues and relative allergenic potency.

Some investigators have opted to use model nucleophiles to investigate the reactivity of chemicals towards proteins. Model nucleophiles such as butylamine, imidazole and propanethiol were used to represent lysine, histidine and cysteine (respectively) in studies investigating the reactivity of γ,γ-dimethyl-γ-butyrolactone derivatives (48), hex-1--ene sultone and hexane-1,3-sultone (49) and 5-chloro-2-methyl-4-isothiazolin-3-one (MCI) and 2-methyl-4-isothiazolin-3-one (MI) (50).

Irrespective of which protein/peptide model is chosen for study, it is important to also study the variable of pH, as this can affect the reactivity of nucleophiles and electrophiles in different tissue environments. To date, the variable of pH has not been investigated extensively.

Mechanisms of protein binding

The 3 most common reaction mechanisms forming covalent bonds and predicted to be involved in sensitization are nucleophilic substitution on a saturated centre, nucleophilic substitution on an unsaturated centre and nucleophilic addition (5, 51) (Fig. 4). Other reactions, such as electrophilic substitution, have been demonstrated (27). Radical reactions are also thought to be responsible for the formation of strong bonds between haptens and proteins (52). Formation of radicals in

homolytic reactions requires a radical inducer (ultraviolet radiation or molecular oxygen) and involves the cleavage of weakly polar or non-polar bonds. The resulting radical species are uncharged groups of atoms containing an uneven number of electrons. These are highly unstable, reactive species able to modify proteins. ACD caused by nickel, chromium and palladium is also thought to occur through protein binding (53). Cations form co-ordination bonds with nucleophilic centres in the proteins, comparable in strength to covalent bonds. Depending on the metal and its oxidation state, several geometries for co-ordination complexes are possible (tetrahedral, square planar, trigonal bipyramidal and octahedral). However, ACD resulting from metal ions may be caused by mechanisms other than protein – hapten binding (5, 54, 55).

Regardless of the protein/peptide models used, these investigations provide insights into often complicated chemistries involved in modification of proteins/peptides. Particularly useful are comparative studies of chemically related sensitizers with differing potencies.

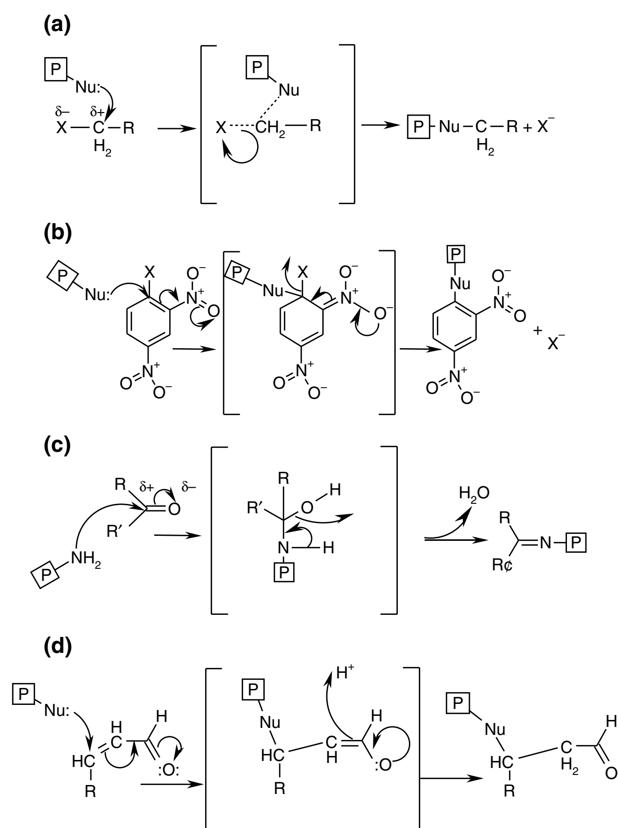

Fig. 4. Common predicted reaction mechanisms between haptens and proteins: (a) nucleophilic substitution on a saturated centre, (b) nucleophilic substitution on an unsaturated centre, (c) Schiff base formation, (d) 1,4-Michael addition. X, leaving group; Nu, nucleophile; P, protein.

For example, HSA and (49) model nucleophiles (Fig. 5a) were used to establish binding mechanisms of hex-1-ene sultone and hexane-1,3-sultone (Fig. 5b), a strong and moderate sensitizer, respectively (41, 49). Alkenesultones are more potent sensitizers *in vivo* than alkanesultones, which was thought to be due to electron-deficient double bond in alkenesultones. Both chemicals were shown to react via nucleophilic substitution at position 3 with model nucleophiles used; only propanethiol and imidazole reacted with hex-1-ene sultone via 1,4-Michael addition reaction at position 2. Incubated with HSA, both molecules reacted with Tyr residues via nucleophilic substitution at position 3. Unlike hex-1-ane sultone, hex-1-ene sultone reacted with a single Lys residue (Lys 199 of HSA), initially at position 3, via nucleophilic substitution followed by an intramolecular Michael addition at position 2, forming aziridinium intermediates which were subsequently hydrolysed to form amino alcohol derivative as the final product. Although the addition of a double bond increases overall reactivity, the initial reaction does not take place at the double bond. The differential potency of the 2 sultones seems to be related to the ability to modify Lys residues selectively.

Similarly, in an earlier study (48, 56), butylamine was again used as a model nucleophile to investigate the binding of α-(ω-substituted-alkyl)-γ,γ-dimethyl-γ-butyrolactones (Fig. 6), using ^{13}C NMR. If the alkyl substituent was a methyl group, these compounds reacted with butylamine via a 2-stage elimination – Michael addition reaction. If alkyl substituents were ethyl or propyl groups, the reaction with butylamine was found

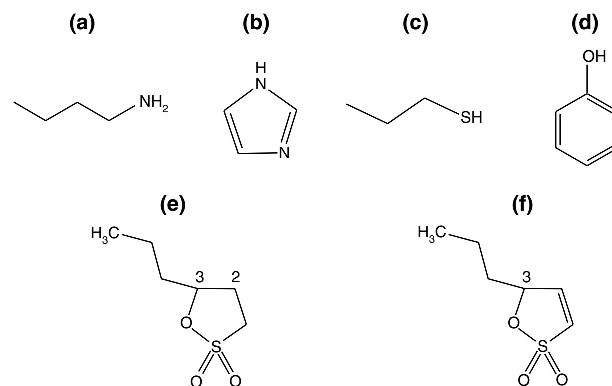

Fig. 5. Examples of the model nucleophiles (a) butylamine, (b) imidazole, (c) propanethiol and (d) phenol used to represent the reactive groups in amino acids lysine, histidine, cysteine and tyrosine, respectively, for biochemical analyses of nucleophile–hapten conjugates. Structures of hex-1-ene-1,3-sultone, (e) and hexane-1,3-sultone, (f) which reacted with model nucleophiles and human serum albumin (HAS) (41, 49).

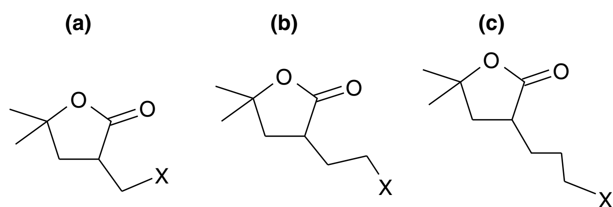

Fig. 6. Structures of α-(ω-substituted-alkyl)-γ,γ-dimethyl-γ-butyrolactones (X, leaving group). (a) alkyl = methyl, (b) alkyl = ethyl, (c) alkyl = propyl.

to be a single-stage substitution reaction. The substitution reaction is slower than the elimination – Michael addition, indicating that methyl-substituted compounds are stronger sensitizers. These results were compared with guinea-pig skin sensitization test results which confirm that group A (methyl substituent) compounds were indeed much stronger sensitizers than compounds from groups B and C (ethyl and propyl substituents, respectively).

However, depending on the models used, different conclusions can be made about the chemical reactivity of certain chemical entities.

For example, MCI reactions with different models have been characterized in several studies. MCI is an extreme sensitizer (34, 57) and a constituent of Kathon CG (58, 59), a microbiocide used as a preservative in skin care products. The reactions of MCI have been investigated with model nucleophiles (50), model peptide, glutathione (GSH) (44) and HSA (42). The proposed reaction of MCI with nucleophiles confirmed by ^{13}C NMR is shown in Fig. 7. This reaction was shown to take place with imidazole (His in model peptide and HSA studies), whereas reaction with butylamine (Lys in model peptide and HSA studies) required a prior activation with thiol, which gave adducts of amide and thioamide type (Fig. 8).

Similarly, the chemistry of benzoquinone also depends on the model used. 2 recent studies have shown that peptides containing relevant nucleophiles may not always represent good models. The study that utilized model peptide PHCKRM showed that benzoquinone reacted with Cys exclusively (45, 46), whereas the study that utilized cytochrome *c* as a model protein showed that benzoquinone reacted with 2 adjacent Lys residues resulting in cyclized diquinone product (26). Which of the 2 modifications demonstrated by the above studies represents the immunogenic entity remains to be shown.

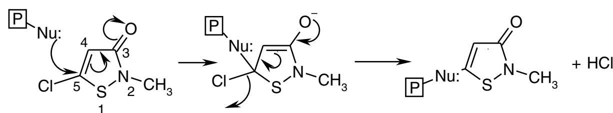

Fig. 7. Proposed mechanism for binding of 5-chloro-2-methyl-4-isothiazolin-3-one (MCI)–1,4-Michael addition at the C-5 position followed by chlorine elimination; Nu, nucleophile; P, protein.

In a study unrelated to skin sensitization, another model peptide (AcGKOMe) was used to examine mechanistic aspects of modification of the ε-amino group of lysine by 2-alkenals (secondary products of lipid peroxidation, implicated in cytotoxicity) (23). The reaction products were characterized using a combination of ^{1}H and ^{13}C NMR, high performance liquid chromatography (HPLC); fast acid bombardment-mass spectrometry (FAB-MS); liquid chromatography-mass spectrometry (LC-MS); matrix assisted laser desorption/ionisation-mass spectrometry (MALDI-MS). The results indicate that 1 ε-amino group of lysine can react with 2 alkenals to form a dihydropyridine ring, and this can further react to form pyridinium moieties or stable pyridinium cross-links.

Complex adducts to proteins of more than 1 molecule of the reacting chemical are not uncommon. Could creation of such elaborate adducts result in a more vigorous immune response?

New Insights

Previously, studies have been focused on 1 or 2 specific chemistries, with the aim to prove a theoretical mechanism for a particular chemical, but inevitably the situation is shown to be more complex than initial theory predicted. Studies on protein haptenation can now employ a combination of classical protein chemistry techniques with modern proteomic tools to look at a spectrum of complex reactivities simultaneously. Mass spectrometry is increasingly being used to determine the extent and exact localization of protein modifications by haptens. A combination of tryptic digestion of modified protein with MALDI-MS peptide fingerprinting and ES-MS/MS analysis of selected suspect modified peptides is proving to be the most successful method.

For example, proteomic techniques were used to investigate the potential of known skin sensitizers, non-sensitizers and irritants to covalently modify model proteins [HSA, human recombinant keratin 14 (K14) and human recombinant cofilin] and peptide [N-terminal part of human globin as a model peptide (sequence VLSPADKTNWGHEYRM FCQIG)] (manuscript in preparation). The data obtained from these experiments have demonstrated that covalent binding is a characteristic of known sensitizers under standardized conditions but with one exception. Sensitizers 2,4-dinitro-1-chlorobenzene (DNCB), MCI and phenyl salicylate were all found to be covalently bound to

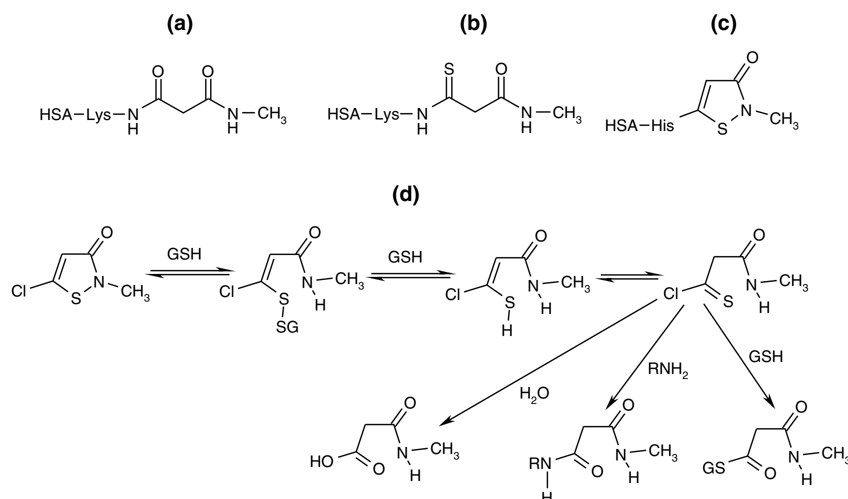

Fig. 8. Proposed 4-[^{13}C]-5-chloro-2-methyl-4-isothiazolin-3-one (MCI) adducts to human serum albumin (HSA): (a) MCI–Lys (Δ mass 100 Da), (b) MCI–Lys (Δ mass 116 Da), (c) MCI–His (Δ mass 114 Da) (all mass shifts calculated include a ^{13}C label). (d) Additional proposed reaction mechanism of MCI with GSH (thiol) and further reactions of thioamide product with water, amines and thiol (59).

HSA. Additionally, covalent binding was demonstrated for DNCB with K14 and human cofilin. No evidence of covalent binding could be found for the non-sensitizers and irritants incubated with model proteins, which is as would be expected. Similarly, evidence of covalent binding to model peptide was observed for all tested sensitizers except MI (non-chlorinated structural relative of MCI). The affinity of MI was previously reported only for Cys of GSH (44), but the lack of evidence for covalent binding may be due to a rapid reaction with thiols yielding unstable products. Conversely, compounds characterized as non-sensitizers *in vivo* (DCNB and benzaldehyde) have demonstrated evidence of covalent binding to the model peptide. This may indicate that these chemicals indeed have sensitizing potential but are extremely weak allergens and do not manifest any response *in vivo*. Indeed, DCNB has been reported as a weak sensitizer (60). Nevertheless, the initial premise that sensitizers bind and non-sensitizers do not is being challenged with these types of study, and the situation of protein haptenation may be complex.

Sensitizing compounds have shown different affinities for several amino acid residues in HSA. The binding of DNCB, an extreme sensitizer, was promiscuous, including Lys, His, Tyr, Trp, Cys and the N-terminus, whereas MCI, also an extreme sensitizer, preferred His unless activated by a reaction with thiol, when it would also bind to Lys. Phenyl salicylate preferentially reacted with Lys, His and Cys. Aldehydes were found to be very reactive with the N-terminus of the model peptide. Predicted mechanisms of reaction for cinnamaldehyde with nucleophiles (concomitant Schiff base formation and Michael addition) were confirmed. Interestingly, citronellal showed affinity towards Trp, a residue only seen modified by DNCB. The selectivity for amino acids could perhaps play a major role in the determination of immunogenicity of skin sensitizers.

Overall, it was established that the 3-dimensional protein environment is restrictive to chemical modifications. Only some of the numerous Lys, His and Cys residues present in the model protein molecules appear to be modified, indicating that the local pKa of residues susceptible to modifications is influenced by its immediate neighbours and there may also be steric effects preventing reactions to certain residues.

The above results have also demonstrated the sensitivity of the techniques used, particularly in the detection of covalent binding for compounds previously termed *in vivo* non-sensitizers, which could have sensitizing potential, albeit very weak.

These results emphasize the fact that *in vitro* protein haptenation assays must be very carefully designed and interpreted, such that we can be confident that there is no binding; thus, the conclusion of negative sensitization potential can be drawn. Similarly, it is important that results *in vitro* do not yield false positives, predicting a chemical to be a sensitizer from its protein haptenation characteristics, when *in vivo* it is not a sensitizer.

Potential New *In Vitro* Assay Development

Several attempts designing potential screening methods for protein reactivity of chemicals have recently been published. A GSH binding assay using MALDI-MS was positive for GSH

reactivity for 13 of 14 sensitizing chemicals tested, with all non-sensitizers found to be unreactive towards GSH (18). GSH conjugation is often used as a measure of chemical reactivity towards thiol groups in proteins in other areas of toxicology. Results such as these can be interpreted in 2 ways in the context of skin sensitization: (i) 13/14 sensitizers bind to GSH indicating an inherent reactivity towards protein thiols, and hence, protein thiol reactivity is a good indicator of skin sensitization hazard; (ii) GSH in biological tissues is normally a detoxifying agent that 'mops up' toxic xenobiotic chemicals. At some point, a threshold is reached, i.e. all of the GSH is used up in the tissue, and in a concentration dependent fashion, the toxic potential of the chemical is unleashed in the tissue when the threshold is reached. Skin sensitizers are by default toxic and therefore their binding to GSH could be an implicit property of their skin toxicity but may not be specifically relevant to the skin sensitization process *per se*. Nevertheless, reaction with GSH is indicative of toxic potential and incorporation of a quick/cheap GSH assay in a series of protein/peptide reactivity assays would yield useful information. In an attempt to address the issue of whether sensitizing potency can be related to peptide reactivity kinetics, a peptide reactivity assay was performed using liquid chromatography as the analytical tool measuring the depletion of reactive peptide following short incubation with sensitizers (17). No attempt was made to identify and characterize the resulting adducts. The peptides were designed to have only 1 reactive site (sequence AcRFAAXAA, X = K, C or H), and the pH of the incubating medium was set at the pKa of the reactive amino acid side chain, thus maximizing the reaction potential. Good correlations between the extent of binding and sensitizing potency (as determined in the LLNA) were shown for a large set of compounds incubated with peptide containing Cys and Lys, but not His. Further promising results were obtained with the addition of a simple metabolizing step (Gerberick, F., personal communication). However, given that the reaction is only possible with 1 nucleophile at the time, this assay would not detect the activation of MCI by thiol and the subsequent covalent modifications of Lys (42). Similarly, benzoquinone reactions with Cys and Lys individually would be apparent in this assay, but the complex reaction with 2 adjacent Lys residues and subsequent cyclization [as observed in reaction with cytochrome *c* (26)] would not. However, we should reiterate that we are unsure of the immunological relevance of such observed modifications.

Another way of detecting protein haptenation by skin sensitizers is through the use of standard enzyme-linked immunosorbent assay (ELISA) methodology. Particular advantages of an ELISA approach are (i) the simplicity and discrete stepwise nature of the assay design for macromolecules and peptides; (ii) simultaneous analyses of multiple treatment conditions and incorporation of a breadth of chemistries; (iii) the potential for higher throughput technologies to be used and (iv) the suitability for the addition of a discrete metabolic pre-step to activate prohaptens appropriately prior to a protein modification step. After an initial promising pilot study using HSA as a model protein (unpublished data) we began further optimization of an ELISA test system with a view to assaying a larger number of chemicals and ultimately incorporating a metabolic pre-step. This assay could also be used with short, specifically designed peptides containing relevant amino acids to cover a breadth of reactive chemistries, with antibodies specific for the designed peptides.

Conclusions and Future Directions

The ultimate goals within the skin sensitization field are to develop better *in vitro*/*in silico* tools for higher throughput screening of chemicals for skin sensitization hazard in early product development and to devise novel, alternative approaches to replace existing *in vivo* assays for predicting sensitization hazard and potency in humans (61). Better understanding of the skin sensitization process at both the cellular and molecular levels will help to support this goal.

Recent advances in understanding the molecular basis of skin sensitization and the potentially associated chemistries are providing more evidence for the covalent protein haptenation theory but are also showing additional mechanistic complexity. Recently, further insights have become available in terms of the cellular localization of hapten–protein binding, and further studies in this area would be informative. The selectivity of haptenation for cellular and extracellular proteins with different types of chemicals (contact and respiratory allergens) was recently investigated (62). When incubated with cells and serum together, contact sensitizers were found to selectively bind to cellular proteins, as opposed to respiratory sensitizers which selectively bound to serum proteins. It would be interesting to explore the cellular locality of protein haptenation further (5). Additionally, advances in cutaneous functional proteomics are providing critical data in our understanding of skin

sensitization (63). This may help us determine the immunologically relevant proteins which are targets for sensitizing molecules. Better predictive assays could be designed if particular types of modifications were found more immunogenic than others. Equally better model proteins/peptides could be chosen if we were able to find out what exactly is presented to the naïve T cell associated with MHC II. It is at least theoretically possible to isolate the MHC II molecules and elute the peptides attached via hydrogen bonds using acid elution (64–68). Sophisticated ultra-sensitive mass spectrometry techniques would allow for unambiguous identification of the peptide sequence and any modifications present on them. If covalently modified peptides are shown to be immunogenic entities in contact sensitization, this could be of great use in the development of predictive assays. Peptide sequence(s) could then also be used to identify the intact proteins involved in sensitization, further aiding the choice of model proteins or design of model peptides for predictive purposes.

It is difficult to envisage that an accurate, single *in vitro* method of prediction could be developed as there are many factors contributing to the manifestation of ACD, including exposure level, chemical structure, lipophilicity, protein binding affinity and specificity, immune responsiveness, etc. It is more likely that a novel multi-component tiered testing strategy or risk assessment approach could be derived that would need to be validated against the *in vivo* potency data from LLNA and human data for known sensitizing chemicals.

Any testing strategy should be designed to minimize both false positives and false negatives by including as broad a range of mechanisms/chemistries as can be identified. It is likely that any testing strategy can never be 100% predictive; hence, it is also important to devise new strategies in the light of how the data will be used for making the ultimate prediction on whether a chemical will act as a sensitizer or not. Given the uncertainties inherent in predicting *in vivo* outcomes from *in vitro* technologies, it could be that the ultimate outcome may be probabilistic in nature rather than deterministic.

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