Session 5.03
Progress and needs for developing and validating alternatives for dermal sensitisation testing

Dendritic Cell Research Projects

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Introduction

The sensitising potential of chemicals is usually identified on the basis of animal studies. In addition to increasing public concern regarding the use of animal testing for the toxicological evaluation of new chemicals, the adoption of the 7th Amendment to the Cosmetics Directive in Europe will result in animal testing of ingredients used in cosmetics being subjected to severe restrictions by 2009 which will be tightened by 2013. The development of \textit{in vitro} models for predicting the sensitising potential of new chemicals is therefore an obvious need.

\textit{In vitro} sensitisation tests will have to reflect the complex interactions of a chemical with the different compartments of the immune system. COLIPA STTF is therefore supporting a range of research projects reflecting our current understanding of the molecular and cellular events occurring during the sensitisation process from aspects of chemistry, peptide binding and skin metabolism to intracellular signalling pathways induced by allergens (see fig. 1).

The Colipa dendritic cell research projects

Dendritic cells (DCs), whose central role during the induction phase of skin sensitisation is well documented (Enk et al., 1993; Lepoivre et al., 1998; Aiba et al., 1997; Smith and Hotchkiss, 2001), were rapidly perceived as promising \textit{in vitro} test systems. DC research projects supported by STTF evaluated the relevance of IL-1\textbeta mRNA expression or changes in gene expression using gene microarrays for predicting potential sensitisers. Myeloid cell lines are being evaluated as surrogate DCs. Intracellular signal transduction pathways are being explored in order to understand the underlying biological mechanisms.

\textbf{Keywords:} dendritic cells, contact sensitisation, hapten, \textit{in vitro} model
cultures of human DCs for predicting potential sensitisers. In 1998, the STTF decided to support a research project to evaluate the relevance of IL-1β mRNA expression by cultured dendritic cells as an endpoint for predicting potential skin sensitisers. Dendritic cells were cultured from peripheral blood drawn from human volunteers. Although donor specific variation in the phenotype of DCs was observed, all blood-derived DCs constitutively expressed mRNA for IL-1β, IL-6 and IL-18. A significant increase (2-3 fold) in IL-1β mRNA expression was observed with DCs derived from 4 out of 9 donors after in vitro exposure to the known contact sensitisers 2,4-dinitrofluorobenzene (DNFB). The variation in DNFB-induced up-regulation of IL-1β expression appeared to be donor dependent. In no instance did treatment of DCs with the non-sensitising skin irritant sodium lauryl sulphate (SLS) increase IL-1β mRNA expression. Exposure to DNFB was largely without effect on constitutive IL-6 or IL-18 mRNA expression. However, although apparently selective for skin sensitising chemicals, the induced changes in IL-1β expression were modest and it is not known whether less potent sensitising chemicals would have the same ability to influence IL-1β mRNA levels (Pichowski et al., 2000).

This work demonstrated that there is some basis for evaluating IL-1β, and certain other genes, based upon what was understood of their mechanistic relevance in the development of skin sensitisation. However, they may not necessarily represent the most appropriate markers for the identification of skin sensitisers and skin irritants.

The STTF thus decided to support a further project proposal making use of the recent availability of DNA arrays, which permit characterisation of induced changes in gene expression in a more holistic fashion. Using this technology, gene expression changes in peripheral blood-derived dendritic cells were analysed following exposure to a contact allergen. Briefly, peripheral blood-derived DCs were exposed for 24 h to either 1 mM or 5 mM dimethylbenzenesulfonic acid (DNBS). Changes in gene expression were analysed using Affymetrix® gene chips. 1 mM and 5 mM DNBS induced 173, respectively 1249 significant gene changes. Many of the gene expression changes were found

**Molecular and cellular events occurring during sensitisation**

- **The chemical most penetrate the skin and react with endogenous proteins**
- **Haptens are internalised and processed by immature dendritic cells (DCs)**
- **DC activation occurs upon skin contact with immunogenic chemicals and activates DCs to regulate a set of cell surface markers (e.g., CD86, CD40), secrete various cytokines, such as IL-18, and modulate the expression of relevant genes.**
- **Activated DCs migrate from the epidermis into the draining lymph node and complete maturation**
- **Activated DCs present fragments of the haptene self-protein to T-helper cells, resulting in the clonal expansion of antigen-specific T cells and the systemic reactivation of sensitised T lymphocytes.**

**Related Colipa activity for developing in vitro alternatives**

- **Development of a toxicokinetic model for better predicting ocular bioavailability of potential skin allergens, improving significantly the current model in the area of skin penetration kinetics**
  - Creator: (Unit: Cosmesan)
  - Timeframe: 3 years

- **Development of a peptide reactivity assay for predicting skin allergens**
  - Creator: (Unit: Louis Pasteur Strasbourg)
  - Timeframe: 3 years

- **Identification of potential sensitisers by immunological detection of relevant binding to designer peptides**
  - Creator: (Unit: Coesfeld)
  - Timeframe: 2 years

- **Identification of changes in gene expression associated with exposure to skin sensitisers for subsequent examination of their physiological role and their utility as a predictive marker for skin sensitizers**
  - Creator: (Unit: LGTM Sentzu)
  - Timeframe: 3 years

- **Examination of known sensitisation markers in dendritic cell lines and optimisation of such techniques**
  - Creator: (Unit: Coesfeld)
  - Timeframe: 3 years

- **Inter laboratory (using) trial on modulation of human mycobacterium cell lines phenotypes and function by chemicals in ICD**
  - Creator: (Unit: KNO, Turku)
  - Timeframe: 1 year

- **Identification of signal transduction pathways that play essential role in regulation of cell proliferation, differentiation, inflammation and apoptosis. Establishment of a predictive test for the screening of potential allergens and contact sensitizers vs. irritants.**
  - Creator: (Unit: LR/ER, A37-M2, INSEM Lyon, B. Le Vivel (LVMH))
  - Timeframe: 1-3 years

**FP6 “SENS-IT-IV” Integrated Project**

Submission of a solid proposal with cosmetic industry contribution under the 6th FP call. Contribute in progress on research on alternatives for skin and respiratory allergy. Besides supporting the pre-submission phase, COLIPA contributes as participant in the technology transfer and dissemination activities and ensures link with internal research programs.

Involved partners: Avances, RCVAM, BCOIPA, IVTIP, COLIPA, cosmetic, pharma, chemical companies.

Timeframe: 5 years

**Fig. 1: Molecular and cellular events occurring during sensitisation and related Colipa activity for developing in vitro alternatives.**
to be induced only by exposure to DNBS and not by exposure to the non-allergen benzenesulfonic acid (Ryan et al., 2004). These data, in part, provided support of the theory that exposure to contact allergens can induce the maturation of DCs. Additionally, some of the transcript changes identified through this approach may be shown to be sufficiently robust, sensitive and selective, such that they are suitable for use in the development of an in vitro predictive assay (Ryan et al., 2004).

There was also a need for a source of homogeneous and reproducible DCs. STTF thus initiated a research project concerning the analysis of the modulation of human myeloid cell lines’ phenotypes and functions by chemicals in 2002.

Three human myeloid cell lines, U-937, THP-1 and KG-1, were evaluated as a replacement of human blood derived DCs. The cytokine environment (granulocyte-macrophage colony stimulating factor, interleukin-4 (IL-4), stem cell factor, transforming growth factor-β1 and tumour necrosis factor-α (TNF-α)) was first optimised for the induction of differentiation into DC-like cells. TNF-α was used to determine the maximum extent and the kinetics of CD86 modulation. The results indicated that among the three tested lines, IL-4 pre-treated U-937 cells provided the optimal CD86 expression range. In parallel, IL-1β and IL-8 mRNA expression was measured by real time RT-PCR. The modulation of the three markers CD86, IL-1β and IL-8 allowed discrimination of sensitisers from non-sensitisers. Results obtained for sensitisers indicate that a combination of at least two of these markers at more than one time point is needed to establish a reliable classification system (Python et al., 2004).

DCs or DC cell lines seem to be specifically activated by sensitising chemicals. In order to better understand the underlying molecular mechanisms, the STTF decided in 2004 to support a project proposal concerning the exploration of intracellular signal transduction pathways in activated dendritic cells.

Experiments were performed on immature human monocyte-derived dendritic cells, induced to mature by LPS, TNF-α or nickel treatment. The pathways of the main kinases known from literature to be involved in intracellular signalling were investigated by use of specific inhibitors at each step of the phosphorylation cascade. Preliminary results showed that CD86 expression induced by DC maturation is influenced by some of these inhibitors, suggesting an essential role of the respective kinases (Trompezinski et al., 2005).

The Colipa Skin Tolerance Task Force (STTF) is playing a very active role in promoting research on in vitro alternatives to in vivo sensitisation tests. Its DC research programme is a key part of an integrated effort to develop alternatives to animal tests (see fig. 1).

**References**


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Hapten-Protein Binding: What Do We Know?

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Summary

The chemical modification of self skin proteins (protein haptenation) and formation of macromolecular immunogens is one of the key molecular events in skin sensitisation. This widely accepted concept can be used to explain the sensitising capacity of many known skin sensitisers and could be used in in vitro assays to predict the sensitisation potential of a chemical. Here we review our current knowledge, highlight the gaps in our understanding of protein haptenation and discuss how we can use such knowledge in the development of novel alternative in vitro approaches for predicting skin sensitisation potential in the future.

Keywords: skin sensitisation, hapten-protein binding, peptide binding, in vitro assay

Introduction

Allergic contact dermatitis (ACD) is a delayed (type IV) hypersensitivity reaction to an exogenous chemical mediated by T-cell related processes (Girolomoni et al., 2004; Rustemeyer et al., 2004; Coombs and Gell, 1975). However, validated in vitro alternatives for assessment of skin sensitisation potential and estimation of relative potency are not available to date, so these assessments are based on in vivo methods, such as the murine local lymph node assay (LLNA) (Basketter et al., 2001).

Improving our understanding of the cellular and molecular mechanisms of the sensitisation process could result in novel opportunities for development of alternative in vitro methods for assessing skin sensitisation hazard and relative potency of chemicals.

Several approaches are being investigated to expand our understanding of the sensitisation process (reviewed in Casati et al., 2005). Additionally, there are a number of other published cell culture studies where responses to treatment with sensitisers have been investigated, including keratinocyte cultures and co-cultures of dendritic cells with T cells (Krasteva et al., 1996; Rougier et al., 2000; Rougier et al., 1998; Guironnet et al., 2000). Signal transduction pathways in dendritic cells, such as mitogen-activated protein kinases (MAPKs) and NFκB pathways have also been a subject of several investigations in search of reliable markers (Aiba et al., 2003; Boisleve et al., 2005).

Exploring the mechanisms of hapten-protein binding in the early stages of the skin sensitisation process is based on 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 sensitisers, and that they do this through a process of protein haptenation (Dupuis and Benezra, 1982; Landsteiner and Jacobs, 1935).

The “covalent binding” concept

The landmark paper by Landsteiner and Jacobs proposed that the sensitising chemicals are too small to be recognised by the classical immunological mechanisms. Therefore they need to be bound to protein in order to elicit an immune response. Unless already a protein-reactive molecule, a sensitiser may be chemically or metabolically activated prior to or upon cutaneous absorption.

Generally, reactions of chemicals with proteins lead to the formation of bonds of different strengths. 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 (Lepoittevin, 2001).

Despite the suggestions that non-covalent modes of hapten-protein association or modification of the normal self protein processing pathways and consequent creation of “cryptic” epitopes could lead to an immune response (Pichler, 2001), evidence to date indicates that the interaction between the hapten and the protein must result in the formation of a strong bond.

Over the last years, the methodology (immunochemistry, NMR and mass spectrometry) applied to characterise protein-hapten binding has improved substantially, enabling us to investigate the specificity as well as the extent of protein binding using a variety of skin sensitisers. Covalent binding is now widely accepted as a result of investigations of numerous examples of skin sensitisers and their ability to covalently modify proteins. Hapten-protein binding studies have achieved important milestones in our understanding of protein haptenation mechanisms, despite not always being related to skin sensitisation (Ahmed et al., 2005; Baker et al., 1998; Conduah Birt et al., 1998; Nerland et al., 2003; Person et al., 2003; Tracey and Shuker, 1997; Walker, 1976; Fabrizi et al., 2003).
**Considering the chemicals**

Chemicals may react with many different skin proteins at different amino acid sites, but in general protein molecules are rich in nucleophiles and the sensitising chemicals are reactive electrophiles or can be metabolically converted to such. The potential reaction mechanisms that lead to skin sensitisation have been discussed by many authors (Basketter et al., 1995; Smith and Hotchkiss, 2001) but very few of them have been proven experimentally.

**Considering the target**

The main targets of small molecule electrophiles are amino acid side chains with nucleophilic properties. Nucleophiles either have atoms containing one 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 sulphhydryl 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 ionisation, bearing in mind that the skin pH ranges from 5.5 on the stratum corneum surface to physiological 7.4 in the epidermis and dermis (Smith and Hotchkiss, 2001). The ability of a chemical to react with a nucleophile may be hindered or enhanced by the nucleophile’s position in a 3D protein environment.

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 proteomics techniques (Celis et al., 1998). It is logical to assume that a protein reactive chemical will modify any available nucleophile to some extent given suitable conditions. A recent study investigating the binding of cinnamaldehyde to human skin homogenates showed that this moderate sensitiser 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 (Elahi et al., 2004). 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**

A number of different models have been used to study protein haptenation. Human serum albumin (HSA) is often the model of choice for protein binding assays. This is a well characterised protein and around 40% of extravascular HSA is located in the skin (Peters, 1996). Several key HSA residues have been shown to be selectively and covalently modified by chemicals, such as Cys34 (Yasuzawa and Tomer, 1997), Lys 190 (Bohney et al., 1992), Lys 199 (Walker, 1976; Bertucci et al., 1995; Meschkat et al., 2001), His 9, His 146, His 338 (Alvarez-Sanchez et al., 2004) and Arg 410 (Ahmed et al., 2005). HSA has 17 pairs of cysteines involved in disulphide linkages and only one free Cys residue (Cys34). Similarly, there is only one Trp residue on HSA. Other macromolecules with different properties and amino acid composition may reveal different mechanisms of protein haptenation for the same chemical. The question about the immunological relevance of specific residue modifications in macromolecules remains. For example, it has been suggested that a tolerising effect may be due to exclusive modification of sulphhydryl groups (Parker et al., 1983).

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 VLSPADKTNWBGHEYRMFCQIG), was used to investigate binding of 4-chlorobenzenediazoniaion hexafluorophosphate (Tracey and Shuker, 1997), acetaldehyde (Conduah Birt et al., 1998) and 5-chloro-2-methylisothiazol-3-one (Alvarez-Sanchez et al., 2004). Glycine apart, this peptide contains one residue of each of the commonly occurring amino acids, with Cys residue of the peptide often carboxymethylated or simply omitted from synthesis to avoid peptide dimerisation in reactivity studies. Similarly, the synthetic peptide PEPAPKPSKGSKKAVTKAQN was used to investigate reactions of phosgene (Fabrizi et al., 2003).

Synthetic peptides unrelated to any proteins have also been used as models such as N-acetylglycyllysine O-methyl ester (AcGKOMe), which was used to investigate the complex reactivities of 2-alkenals (Baker et al., 1998). Similarly, a synthetic peptide PHCKRM was used to investigate the binding of 1,4-benzoquinone (Ahlfors et al., 2003), 4-r-butyl-1,2-benzoquinone (Hanson et al., 1999) and two metabolites of a prohapten (5R)-5-isopropenyl-1-methyl-1-methylene-2-cyclohexene (Nilsson et al., 2005; Ahlfors et al., 2003).

The major problem with using small peptides is the further step away from the biological conditions in the skin, as the potentially crucial influence of the 3-dimensional protein environment is not represented. However, model peptides have their place in such investigations, proving explanations for chemistries that may currently only be theoretical for many sensitisers and revealing new potential chemistries (Dupuis and Benezra, 1982; Smith and Hotchkiss, 2001). 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 as well as the ability to use quantification.

Some investigators have opted to use model nucleophiles such as butylamine, imidazole and propanethiol representing lysine, histidine and cysteine (respectively) to investigate reactivity of chemicals towards proteins (Franot et al., 1994; Meschkat et al., 2001; Alvarez-Sanchez et al., 2003).

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 sensitisers with differing potencies as illustrated in experiments with hex-1-ene and hexane-1,3-sultones, a strong and moderate sensitiser, respectively (Meschkat et al., 2001) and butyrolactones (Franot et al., 1994).

However, depending on the models used, different conclusions can be made about the chemical reactivity of certain chemical entities. This is illustrated in the series of papers about reactivity of 5-chloro-2-methyl-4-isothiazolin-3-one (MCI) with...
different models. MCI is an extreme sensitisier (Baskettet al., 1999; Gerberick et al., 2004) and a constituent of Kathon CG (Collier et al., 1990a; Collier et al., 1990b), a microbiocide used as a preservative in skin care products. The reactions of MCI have been investigated with model nucleophiles (Alvarez-Sanchez et al., 2003), model peptide, GSH (Alvarez-Sanchez et al., 2004) and HSA (Alvarez-Sanchez et al., 2004). The proposed substitution reaction was confirmed by $^{13}$C NMR and 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.

Similarly, the chemistry of benzoquinone also depends on the model used. The study which utilised model peptide PHCKRM showed benzoquinone reacted with Cys exclusively (Hanson et al., 1999; Ahlfors et al., 2003), whereas the study which utilised cytochrome c as a model protein, showed benzoquinone reacted with two adjacent Lys residues resulting in cyclised diquinone product (Person et al., 2003). Which of the two modifications represents the immunogenic entity remains to be shown.

In a study unrelated to skin sensitisation, another model peptide (N-acetylglucyllysinine O-methyl ester, (AcGKOMe)) was used in a study to examine mechanistic aspects of modification of the ε-amino group of lysine by 2-alkenals (Baker et al., 1998). The reaction products were characterised using a combination of $^1$H and $^{13}$C NMR, HPLC separation, FAB MS, LC-MS and MALDI-MS. The results indicate that one ε-amino group of lysine can react with two alkenals to form a dihyropyridine ring, and this can further react to form pyridinium moieties or stable pyridinium cross-links. Such complex adducts to proteins of more than one molecule of the reacting chemical are not uncommon. Creation of such elaborate adducts could result in a more vigorous immune response.

Conclusions and future directions

The ultimate goals within the skin sensitisation field are to develop better *in vitro/in silico* tools for screening of chemicals for skin sensitisation hazard in early product development and to devise alternative approaches to replace *in vivo* assays for predicting sensitisation hazard and potency in man (Divkovic et al., 2003). Recent advances in understanding the molecular basis of skin sensitisation and the associated chemistries are providing more evidence for the covalent protein haptenation theory but are also revealing additional mechanistic complexity. Further insights have become available in terms of the cellular localisation 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 (Hopkins et al., 2005). When incubated with cells and serum together, contact sensitisers were found to selectively bind to cellular proteins, as opposed to respiratory sensitisers which selectively bound to serum proteins. It would be interesting to explore the cellular locality of protein haptenation further (Smith and Hotchkiss, 2001). Additionally, advances in cutaneous functional proteomics are providing critical data in our understanding of skin sensitisation (Huang et al., 2005). This may help determine the immunologically relevant proteins which are targets for sensitising 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 found 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 (Hayden et al., 1996; Lippolis et al., 2002; Sharif et al., 2003; Suri et al., 2003; Park et al., 2003). Sophisticated ultra sensitive mass spectrometry techniques would allow unambiguous identification of the peptide sequence and any modifications present. If covalently modified peptides are shown to be immunogenic entities in contact sensitisation 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 sensitisation, further aiding the choice of model proteins or design of model peptides for predictive purposes (Divkovic et al., 2005).

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The Chemistry of Skin Allergy

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Summary
Allergic Contact Dermatitis (ACD) is a very common disease resulting from epidermal proteins being chemically modified by haptens. The processing of such modified proteins by Langerhans cells, the main antigen-presenting cells in the epidermis, generates altered peptides that are subsequently presented, in association with MHC molecules, to naïve T-lymphocytes in the lymph nodes. The whole process results in the selection and activation of T-lymphocyte sub-populations with T-cell Receptors (TcR) specific for the chemical modification. Haptens are usually low molecular weight molecules, lipophilic enough to penetrate the epidermis through the stratum corneum, and with a potent chemical reactivity allowing the formation of a covalent link with nucleophilic residues on protein amino acid side chains. For some time it was considered that the more a molecule is able to modify proteins, the better a sensitiser it is, and that a direct relation could be established between the overall chemical reactivity of a molecule and its sensitising potential. Today, it is hypothesised that the sensitising potential of a molecule is related to its chemical reactivity towards a few specific amino acids relevant to the sensitisation process. Haptens can modify proteins by many different mechanisms, from classical nucleophilic-electrophilic reactions to radical reactions. The knowledge of how haptens can modify proteins is the basis for the development of predictive alternative tests aimed at the identification of hazard and potency, such as Structure Activity Relationships (SAR), Quantitative Structure Activity Relationships (QSAR) and peptide reactivity tests.

Keywords: allergic contact dermatitis, haptens, pro-haptens, pre-haptens

Introduction
Allergic contact dermatitis (ACD) is a very common disease resulting from epidermal proteins being chemically modified by haptens (Lepoittevin et al., 1998). The processing of such modified proteins by Langerhans cells, the main antigen-presenting cells in the epidermis, generates altered peptides that are subsequently presented, in association with MHC molecules, to naïve T-lymphocytes in the lymph nodes. The whole process results in the selection and activation of T-lymphocyte sub-populations with T-cell receptors (TcR) specific for the chemical modification. Haptens are usually low molecular weight molecules, lipophilic enough to penetrate the epidermis through the stratum corneum and with a potent chemical reactivity, allowing the formation of a covalent link with nucleophilic residues on protein amino acid side chains. For some time it was considered that the more a molecule can modify proteins, the better a sensitiser it is, and that a direct relation could be established between the overall chemical reactivity of a molecule and its sensitising potential. Today, it is hypothesised that the sensitising potential of a molecule is related to its chemical reactivity towards a few specific amino acids relevant to the sensitisation process. Haptens can modify proteins by many different mechanisms, from classical nucleophilic-electrophilic to radical reactions. The knowledge of how haptens can modify proteins is the basis for the development of predictive alternative tests aimed at the identification of hazard and potency, such as Structure Activity Relationships (SAR), Quantitative Structure Activity Relationships (QSAR) and peptide reactivity tests.

Main electrophilic chemical groups present in contact allergens
Many chemical groups have electrophilic properties and are thus able to react with various nucleophiles to form covalent bonds. Table 1 shows those chemical groups most frequently found in contact allergens.
If we consider biological systems from a chemical viewpoint, it becomes apparent that a very large proportion of structures, especially nucleic acids and proteins, contain many electron-rich groups (those containing nitrogen, phosphorus, oxygen or sulphur). We can thus consider biological systems as being overall nucleophilic. It is therefore not surprising that many biological mechanisms are disturbed on contact with electrophilic chemical substances. Depending on the site of action of these electrophilic molecules, the effect can be mutagenic (Frierson et al., 1985), toxic (Guengerich and Liebler, 1985), or allergenic if the target is the epidermis. In proteins, the side chains of many amino acids contain electron-rich groups capable of reacting with allergens (fig. 2). Lysine and cysteine are those most often cited, but other amino acids containing nucleophilic heteroatoms, such as histidine, methionine, and tyrosine, can react with electrophiles (Means and Feeney, 1971). Thus, it has been shown by nuclear magnetic resonance (NMR) studies that nickel sulphate, for example, interacts with histidine residues of peptides (Romagnoli et al., 1991) and that methyl alkane-sulphonates, allergenic methylating agents, mainly react with histidine and to a lesser extent with lysine, methionine, cysteine, and tyrosine (Lepoittevin and Bezerra, 1992). If we consider the
chemical structure of some allergens (fig. 2) in the light of the chemical principles already outlined, it is easy to understand that all of these molecules will be able to react with biological nucleophiles. The so formed extremely stable covalent bonds could then lead to the triggering of delayed hypersensitivity.

Chemical selectivity of haptens for amino acids

A direct consequence of the diversity of hapten-protein interactions is the existence of selectivity for amino acid modifications. For example, we have shown that the α-methylene-γ-butyrolactones, the major allergens of plants of the Asteraceae family, principally modify lysine residues (Franot et al., 1993). It has also been shown that not all modifications were antigenic and that the sensitisation potential of a molecule is probably more related to its ability to modify some specific residues rather than to modify a large number of amino acids. Thus, the difference in

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<td>Metal salts</td>
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Tab. 1: Main electrophilic groups seen in contact allergy with the products formed.

Fig. 1: Principal nucleophilic residues in proteins.

Fig. 2: Examples of sensitising molecules. The electrophilic centre is indicated by an arrow.
sensitising potential of two sultone derivatives, an alkenylsul- 
tone (a strong sensitiser) and an alkylsultone (a weak sensitiser),  
which differ only by the presence of a double bond, could rather  
be explained by the selective modification of lysine residues by  
the strong sensitiser than by the many tyrosine residues modified  
by both derivatives (Meschkat et al., 2001a, 2001b).  

A direct consequence of hapten selectivity for amino acids is  
the existence of differences in the modification sites of pro- 
teins and in the density of these modifications. These differ- 
ces, initially purely chemical, seem increasingly to have a  
major impact on the response of the immune system. The  
selectivity of the sites of haptenisation is directly involved in  
the selection of the peptide fragments that are presented by the  
APC to the T cells and thus in the selection of T cell receptors.  
This selectivity also indirectly controls the level of haptenisa- 
tion of the protein or proteins. It appears that the epitope den- 
sity on the surface of the APC directly or indirectly directs the  
immune response towards Th1 or Th2, high epitope densities  
directing the response towards Th1 and low densities towards  
Th2 (Hosken et al., 1995). It is reasonable to ask if this selec- 
tion of response profile, related to epitope density and thus to  
hapten reactivity, might not explain, for example, the observed  
differences between respiratory and skin allergens.  

In recent years, the radical mechanism has gained increased  
interest in the discussion of the mechanism of hapten-protein  
binding. This mechanism, which has never been firmly estab- 
lished, has been postulated to explain, for example, the aller- 
genic potential of eugenol versus iso-eugenol (Barratt, 1992).  
More recently, studies indicating that radical reactions were  
important for haptenings containing allylic hydroperoxide groups  
have been published (Lepoittevin and Karlberg, 1994;  
Giménez-Arnau, 2002).

**Metabolism and pro-hapten**

Far from being an inert tissue, the skin is the site of many  
metabolic processes, which can result in structural modifica- 
tion of the xenobiotics that penetrate it (Hotchkiss, 1998).  
These metabolic processes, primarily intended for the elimina- 
tion of foreign molecules during detoxification, can, in certain  
cases, convert harmless molecules into derivatives with elec- 

trophilic, and therefore allergenic, properties. The metabolic  
processes are mainly based on oxidation reactions via  
extremely powerful enzymatic hydroxylation systems, such as  
the cytochrome P450 enzymes (Mansuy, 1985), but  
monooxynine oxidases, which convert amines to aldehydes, and  
peroxidases seem to play an important role in the metabolism  
of hapten. When activated by the production of hydrogen per- 

oxide during the oxidative stress following the introduction of  
a xenobiotic into the skin, peroxidases convert the electron- 
rich aromatic derivatives (aminated or hydroxylated) into  
quinones, which are powerful electrophiles. In this way, it has  
been proposed that the long-chain catechols, responsible for  
the severe allergies to poison ivy (*Rhus radicans* L.) and  
poison oak (*Rhus diversiloba* T.), could be oxidised in vivo to the  
highly reactive orthoquinones (Dupuis, 1979) (fig. 3). The  

same applies to paraphenylenediamine or hydroquinone  
derivatives, such as the allergens from *Phacelia crenulata* Torr.  
(Reynolds and Rodriguez, 1981), which are converted into  
electrophilic paraquinones. Metabolic reactions involving  

enzymatic hydrolys can also occur in the skin. It is thus that  
the tuliposides A and B, found in the bulb of the tulip (*Tulipa  
gesneriana* L.), are hydrolysed, releasing the actual allergens,  
tulipalin A and B (Bergmann et al., 1967).  

All these molecules, which have themselves no electrophilic  
properties and cannot therefore be haptens but which can be  
metabolised to hapten, are referred to as pro-hapten (Landsteiner and Jacobs, 1936; Dupuis and Benzer, 1982) and  
play an important role in contact allergy because of their num- 
ber and their highly reactive nature. The fact that the structure  
of the metabolised molecule can be far removed from the struc- 
ture of the initial molecule can make allergologic investiga- 
tions even more difficult.

**Air oxidation and pro-hapten**

Non-enzymatic processes, such as reaction with atmospheric  
oxogen or ultraviolet irradiation, can also induce changes in  
the chemical structure of molecules. Many terpenes sponta- 
neously auto-oxidise in air, producing allergising derivatives.  
In the 1950s it was found that allergenic activity of turpentine  
was mainly due to hydroperoxides of the monoterpen  
δ-carene (Hellerstrom et al., 1955). This is also the case for  
abiatic acid, the main constituent of colophony, which is con- 
verted into the highly reactive substance hydroperoxide  
(Karlbeg, 1988) by contact with air (fig. 4). Such an auto-oxi- 
dation mechanism has also been demonstrated for another  
monoterpen, *d*-limonene, found in citrus fruits. *d*-Limonene  
itself is not allergenic, but upon exposure to air hydperox- 
ides, epoxides, and ketones are formed that are strong allergens  
(Karlbeg et al., 1994).

![Fig. 3: Examples of pro-hapten metabolism.](image-url)
All these molecules, which have themselves no electrophilic properties and cannot therefore be haptens but which can be oxidised into haptens, are referred to as pre-haptens.

**Conclusion**

The principles that we have just discussed permit a rational approach to the phenomena of contact allergy, but, in actual fact, we often have available only indirect evidence suggestive of one mechanism or another. Although the chemical basis for hapten-protein interactions can be checked in the laboratory by the use of nucleophilic amino acids, small peptides and model proteins, and although a certain number of steps can be checked, at the present time no method is available to follow a hapten step by step during the entire immunological process leading to contact allergy. Many points await investigation, but in many cases a "chemical" analysis of the problem does allow us to understand and to foresee cross-allergies and thus to warn the patient about structurally related products.

**References**


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