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

Dendritic Cell Research Projects

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Summary

The Colipa Skin Tolerance Task Force (STTF) is playing an active role in promoting refined methods for in vivo sensitisation tests. Dendritic cells (DCs), whose central role during the induction phase of skin sensitisation is well documented, were perceived as promising in vitro test systems. DC research projects supported by STTF evaluated the relevance of IL-1β 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.

Keywords: dendritic cells, contact sensitisation, hapten, in vitro model

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 *in vitro* models for predicting the sensitising potential of new chemicals is therefore an obvious need. *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; Lepoittevin et al., 1998; Aiba et al., 1997; Smith and Hotchkiss, 2001), were rapidly perceived as promising *in vitro* test systems. The publication of a paper describing specific *in vitro* upregulation of IL-1β mRNA in skin DCs exposed to sensitisers (Reutter and Jaeger, 1997) convinced STTF to initiate a research project aimed at evaluating the relevance of IL-1β mRNA expression in

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 sensitiser 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 dinitrobenzenesulphonic 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

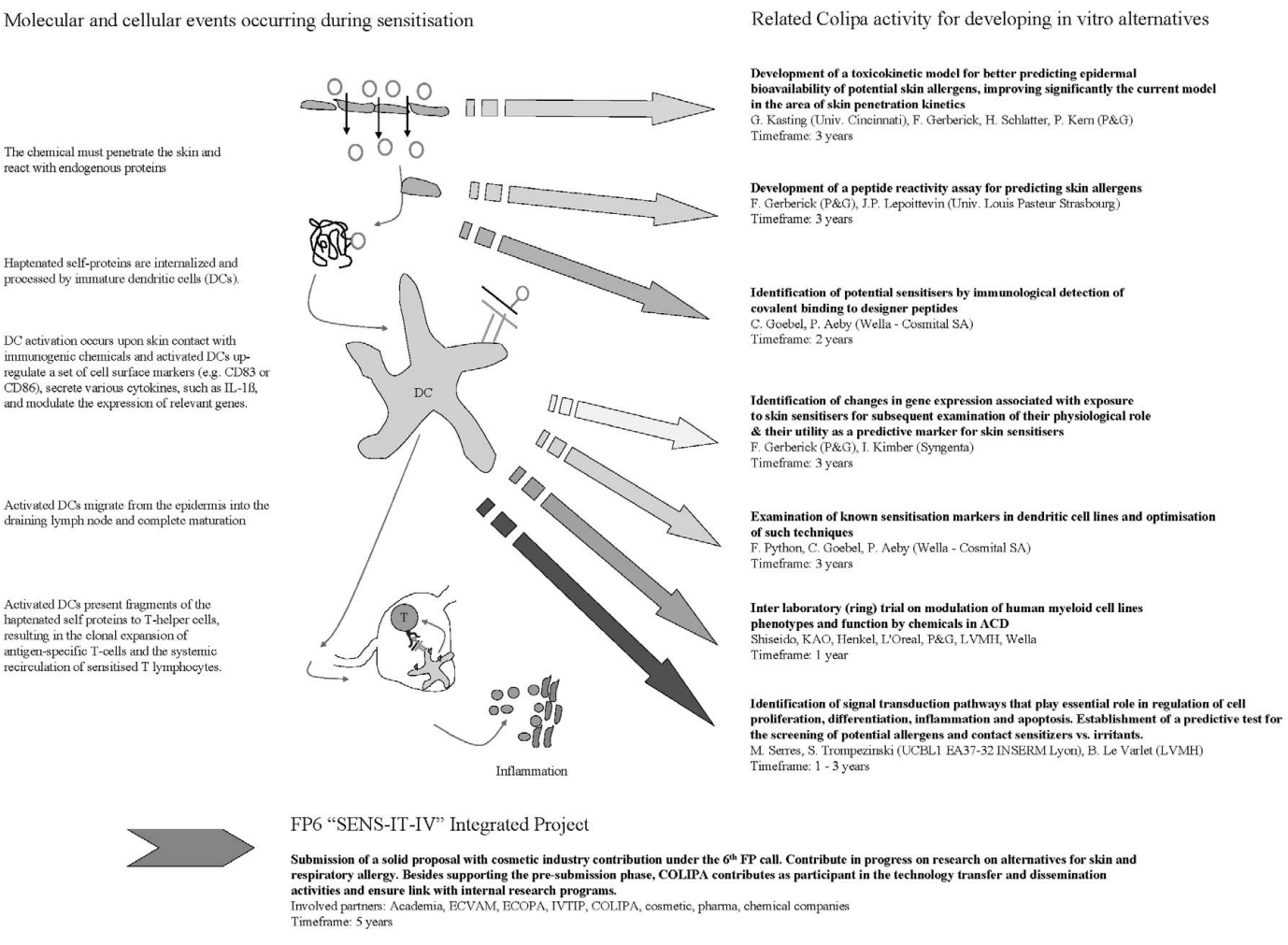

Fig. 1: Molecular and cellular events occurring during sensitisation and related Colipa activity for developing *in vitro* alternatives.

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

Aiba, S., Terunuma, A., Manome, H., and Tagami, H. (1997). Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. *Eur. J. Immunol. 27*, 3031-3038.

Enk, A. H., Angeloni, V. L, Udey, M. C. and Katz, S. I. (1993). An essential role for Langerhans cell-derived IL-1 beta in the initiation of primary immune responses in skin. *J. Immunol. 150*, 3698.

Lepoittevin, J. P., Basketter, D. A., Goossens, A. and Karlberg, A-T. (ed.) (1998). *Allergic Contact Dermatitis – The Molecular Basis*. Berlin: Springer-Verlag.

Pichowski, J. S., Cumberbatch, M., Dearman, R. J. et al. (2000). Investigation of induced changes in interleukin 1ß mRNA expression by cultured human dendritic cells as an in vitro approach to skin sensitization testing. *Toxicol. In vitro 14,* 351-360.

Python, F., Goebel, C. and Aeby, P. (2004). Evaluation and optimization of an in vitro culture system for the identification of contact allergenes, Abstract from the ICTX 2004. *Toxicol. Appl. Pharmacol. 197(3)*, 182.

Reutter, K. and Jaeger, D. (1997). In vitro model for contact sensitization: II. Induction of IL-1ß mRNA in human blood-derived dendritic cells by contact sensitizers. *Toxicol. In vitro 11*, 619-626.

Ryan, C. A., Gildea, L. A., Hulette, B. C. et al. (2004). Gene expression changes in peripheral blood-derived dendritic cells following exposure to a contact allergen. *Toxicol. Lett. 150(3)*, 301-316.

Smith, C. K. and Hotchkiss, S. A. M. (eds.) (2001). *Allergic Contact Dermatitis – Chemical and Metabolic Mechanisms. Chapter 1: Allergic Contact Dermatitis to Small Molecule Xenobiotics.* London and New York: Taylor & Francis.

Trompezinski, S., Denoeud, J., Zabawski B. et al. (2005). NF-κB, PI3K and JNK are essential signaling pathways implicated in human dendritic cells maturation after contact with allergens. Abstract from the 5th World Congress on Alternatives & Animal Use in the Life Sciences, Berlin 2005.

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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 sulphydryl 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 VLSPADKTNWGHEYRMFCQIG), was used to investigate binding of 4-chlorobenzenediazonium 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 PEPAK-SAPAPKKGSKKAVTKAQK 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 reactions 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-*t*-butyl-1,2-benzoquinone (Hanson et al., 1999) and two metabolites of a prohapten (5R)-5-isopropenyl-2-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 sensitiser (Basketter et 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-acetylglycyllysine *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).

References

Ahlfors, S. R., Sterner, O. and Hansson, C. (2003). Reactivity of Contact Allergenic Haptens to Amino Acid Residues in a Model Carrier Peptide, and Characterisation of Formed Peptide-Hapten Adducts. *Skin Pharmacol. Appl. Skin Physiol. 16*, 59-68.

Ahmed, N., Dobler, D., Dean, M. and Thornalley, P. J. (2005). Peptide mapping identifies hotspot site of modification in human serum albumin by methylglyoxal involved in l igand binding and esterase activity. *J. Biol. Chem. 208*, 5724-5732.

Aiba, S., Manome, H., Nakagawa, S. et al. (2003). p38 Mitogen-activated protein kinase and extracellular signal-regulated kinases play distinct roles in the activation of dendritic cells by two representative haptens, NiCl2 and 2,4-dinitrochlorobenzene. *J. Invest. Dermatol. 120*, 390-399.

Alvarez-Sanchez, R., Basketter, D. A., Pease, C, and Lepoittevin, J. P. (2004). Covalent binding of the 13C-labeled skin sensitizers 5-chloro-2-methylisothiazol-3-one (MCI) and 2-methylisothiazol-3-one (MI) to a model peptide and glutathione. *Bioorg. Med. Chem. Lett. 14*, 365-368.

Alvarez-Sanchez, R., Basketter, D. A., Pease, C., and Lepoittevin, J. P. (2003). Studies of chemical selectivity of hapten, reactivity, and skin sensitization potency. 3. Synthesis and studies on the reactivity toward model nucleophiles of the 13C-labeled skin sensitizers, 5-chloro-2-methylisothiazol-3-one (MCI) and 2-methylisothiazol-3-one (MI). *Chem. Res. Toxicol. 16*, 627-636.

Alvarez-Sanchez, R., Divkovic, M., Basketter, D. A. et al. (2004). Effect of Glutathione on the Covalent Binding of the (13)C-Labeled Skin Sensitizer 5-Chloro-2-methylisothiazol-3-one to Human Serum Albumin: Identification of Adducts by Nuclear Magnetic Resonance, Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry, and

Nanoelectrospray Tandem Mass Spectrometry. *Chem. Res. Toxicol. 17*, 1280-1288.

Baker, A., Zidek, L., Wiesler, D. et al. (1998). Reaction of N-acetylglycyllysine methyl ester with 2-alkenals: an alternative model for covalent modification of proteins. *Chem. Res. Toxicol. 11*, 730-740.

Basketter, D. A., Dooms-Goossens, A., Karlberg, A. T. and Lepoittevin, J. P. (1995). The chemistry of contact allergy: why is a molecule allergenic? *Contact Dermatitis 32*, 65-73.

Basketter, D. A., Gerberick, G. F. and Kimber, I. (2001). Measurement of allergenic potency using the local lymph node assay. *Trends Pharmacol. Sci. 22*, 264-265.

Basketter, D. A., Rodford, R., Kimber, I. Et al. (1999). Skin sensitization risk assessment: a comparative evaluation of 3 isothiazolinone biocides. *Contact Dermatitis 40*, 150-154.

Bertucci, C., Ascoli, G., Uccello-Barretta, G. et al. (1995). The binding of 5-fluorouracil to native and modified human serum albumin: UV, CD, and 1H and 19F NMR investigation. *J. Pharm. Biomed. Anal. 13*, 1087-1093.

Bohney, J. P., Fonda, M. L. and Feldhoff, R. C. (1992). Identification of Lys190 as the primary binding site for pyridoxal 5'-phosphate in human serum albumin. *FEBS Lett. 298*, 266-268.

Boisleve, F., Kerdine-Romer, S. and Pallardy, M. (2005). Implication of the MAPK pathways in the maturation of human dendritic cells induced by nickel and TNF-alpha. *Toxicology 206,* 233-244.

Casati, S., Aeby, P., Basketter, D. A. et al. (2005). Dendritic Cells as a Tool for the Predictive Identification of Skin Sensitisation Hazard. *Alternatives to Laboratory Animals 33*, 47-62.

Celis, J. E., Ostergaard, M., Jensen, N. A. et al. (1998). Human and mouse proteomic databases: novel resources in the protein universe. *FEBS Lett. 430*, 64-72.

Collier, P. J., Ramsey, A., Waigh, R. D. et al. (1990). Chemical reactivity of some isothiazolone biocides. *Journal of Applied Bacteriology 69*, 578-584.

Collier, P. J., Ramsey, A. J., Austin, P. and Gilbert, P. (1990). Growth inhibitory and biocidal activity of some isothiazolone biocides. *J. Appl. Bacteriol. 69*, 569-577.

Conduah Birt, J. E., Shuker, D. E. and Farmer, P. B. (1998). Stable acetaldehyde-protein adducts as biomarkers of alcohol exposure. *Chem. Res. Toxicol. 11*, 136-142.

Coombs, R. R. A. and Gell, P. G. H. (1975). Classification of allergic reactions for clinical hypersensitivity and disease. In P. G. H. Gell, R. R. A. Coombs and R. Lachman (eds.), *Clinical Aspects of Immunology* (761-781). London: Blackwell Scientific.

Divkovic, M., Basketter, D. A., Gilmour, N. et al. (2003). Protein-hapten binding: Challenges and limitations for in vitro skin sensitization assays. *Journal of Toxicology – Cutaneous and Ocular Toxicology 22,* 87-99.

Dupuis, G. and Benezra, C. (1982). *Contact Dermatitis to Simple Chemicals: A Molecular Approach*. New York: Marcel Dekker.

Elahi, E. N., Wright Z. M., Hinselwood, D. et al. (2004). Protein Binding and Metabolism Influence the Relative Skin Sensitization Potential of Cinnamic Compounds. *Chem. Res. Toxicol. 17*, 301-310.

Fabrizi, L., Taylor, G .W., Canas, B. et al. (2003). Adduction of the Chloroform Metabolite Phosgene to Lysine Residues of Human Histone H2B. *Chem. Res. Toxicol. 16*, 266-275.

Franot, C., Roberts, D. W., Basketter, D. A. et al. (1994). Structure-activity relationships for contact allergenic potential of gamma,gamma-dimethyl-gamma-butyrolactone derivatives. 2. Quantitative structure-skin sensitization relationships for alpha-substituted-alpha-methyl-gamma,gamma-dimethyl-gamma-butyrolactones. *Chem. Res. Toxicol. 7*, 307-312.

Franot, C., Roberts, D. W., Smith, R. G. et al. (1994). Structure-activity relationships for contact allergenic potential of gamma,gamma-dimethyl-gamma-butyrolactone derivatives. 1. Synthesis and electrophilic reactivity studies of alpha-(omega-substituted-alkyl)-gamma,gamma-dimethyl-gamma-butyrolactones and correlation of skin sensitization potential and cross-sensitization patterns with structure. *Chem. Res. Toxicol. 7*, 297-306.

Gerberick, G. F., Ryan, C. A., Kern, P. S. et al. (2004). A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. *Contact Dermatitis 50*, 274-288.

Girolomoni, G., Gisondi, P., Ottaviani, C. and Cavani, A. (2004). Immunoregulation of allergic contact dermatitis. *J. Dermatol. 31*, 264-270.

Guironnet, G., Dalbiez-Gauthier, C., Rousset, F. et al. (2000). In vitro human T cell sensitization to haptens by monocyte-derived dendritic cells. *Toxicol. In Vitro 14*, 517-522.

Hanson, C., Ahlfors, S. and Sterner, O. (1999). Reactivities of contact allergenic quinones towards a model peptide and identification of the adducts. *Journal of Investigative Dermatology 113*, 320-320.

Hayden, J. B., McCormack, A. L., Yates, J. R., III and Davey, M. P. (1996). Analysis of naturally processed peptides eluted from HLA DRB1*0402 and *0404. *J. Neurosci. Res. 45*, 795-802.

Hopkins, J. E., Naisbitt, D. J., Kitteringham, N. R. et al. (2005). Selective haptenation of cellular or extracellular protein by chemical allergens: association with cytokine polarization. *Chem. Res. Toxicol. 18*, 375-381.

Huang, C.-M., Elmets, C. A., van Kampen, K. R. et al. (2005). Prospective highlights of functional skin proteomics. Mass Spectrom. Rev. e-publication in advance of print.

Krasteva, M., Peguet-Navarro, J., Moulon, C. et al. (1996). In vitro primary sensitization of hapten-specific T cells by cultured human epidermal Langerhans cells – a screening predictive assay for contact sensitizers. *Clin. Exp. Allergy 26*, 563-570.

Landsteiner, K. and Jacobs, J. (1935). Studies on the Sensitisation of Animals With Simple Chemical Compounds. *J. Exp. Med. 61*, 643-656.

Lepoittevin, J. P. (2001). Molecular aspects of allergic contact dermatitis. In R. J. G. Rycroft, T. Menne, P. J. Frosch and J. P. Lepoittevin (eds.), *Textbook of Contact Dermatitis* (59-91). Berlin, Heidelberg: Springer Verlag.

Lippolis, J. D., White, F. M., Marto, J. A. et al. (2002). Analysis of MHC class II antigen processing by quantitation of pep-

tides that constitute nested sets. *J. Immunol. 169*, 5089-5097.

Meschkat, E., Barratt, M. D. and Lepoittevin, J. P. (2001). Studies of the chemical selectivity of hapten, reactivity, and skin sensitization potency. 2. nmr studies of the covalent binding of the (13)c-labeled skin sensitizers 2-[13C]- and 3-[13C]hex-1-ene- and 3-[13C]hexane-1,3-sultones to human serum albumin. *Chem. Res. Toxicol. 14*, 118-126.

Meschkat, E., Barratt, M. D. and Lepoittevin, J. P. (2001). Studies of the chemical selectivity of hapten, reactivity, and skin sensitization potency. 1. Synthesis and studies on the reactivity toward model nucleophiles of the (13)C-labeled skin sensitizers hex-1-ene- and hexane-1,3-sultones. *Chem. Res. Toxicol. 14*, 110-117.

Nerland, D. E., Cai, J. and Benz, F. W. (2003). Selective Covalent Binding of Acrylonitrile to Cys 186 in Rat Liver Carbonic Anhydrase III In Vivo. *Chem. Res. Toxicol. 16*, 583-589.

Nilsson, A. M., Bergstrom, M. A., Luthman, K. et al. (2005). A conjugated diene identified as a prohapten: contact allergenic activity and chemical reactivity of proposed epoxide metabolites. *Chem. Res. Toxicol. 18*, 308-316.

Park, J. H., Lee, Y. ., Kim, K. L. and Cho, E. W. (2003). Selective isolation and identification of HLA-DR-associated naturally processed and presented epitope peptides. *Immunol. Invest 32*, 155-169.

Parker, D., Long, P. V. and Turk, J. L. (1983). A comparison of the conjugation of DNTB and other dinitrobenzenes with free protein radicals and their ability to sensitize or tolerize. *J. Invest Dermatol. 81*, 198-201.

Person, M., Monks, T. J. and Lau, S. S. (2003). An Integrated Approach To Identifying Chemically Induced Post-translational Modifications Using Comparative MALDI-MS and Targeted HPLC-ESI-MS/MS. *Chem. Res. Toxicol. 16*, 598-608.

Peters, T. Jr. (1996). *All about Albumin – Biochemistry, Genetics and Medical Applications*. San Diego: Academic Press.

Pichler, W. J. (2001). Predictive drug allergy testing: an alternative viewpoint. *Toxicology 158*, 31-41.

Rougier, N., Redziniak, G., Mougin, D. et al. (2000). In vitro evaluation of the sensitization potential of weak contact allergens using langerhans-like dendritic cells and autologous T cells. *Toxicology 145*, 73-82.

Rougier, N., Redziniak, G., Schmitt, D. and Vincent, C. (1998). Evaluation of the capacity of dendritic cells derived from cord blood CD34+ precursors to present haptens to unsensitized autologous T cells in vitro. *J. Invest Dermatol. 110*, 348-352.

Rustemeyer, T., von Blomberg, B. M., van Hoogstraten, I. M. et al. (2004). Analysis of effector and regulatory immune reactivity to nickel. *Clin. Exp. Allergy 34*, 1458-1466.

Sharif, S., Mallard, B. A. and Wilkie, B. N. (2003). Characterization of naturally processed and presented peptides associated with bovine major histocompatibility complex (BoLA) class II DR molecules. *Anim Genet. 34*, 116-123.

Smith, C. K. and Hotchkiss, S. A. M. (2001). *Allergic Contact Dermatitis – Chemical and Metabolic Mechanisms*. Taylor & Francis, UK.

Suri, A., Walters, J. J., Kanagawa, O., Gross, M. L. and Unanue, E. R. (2003). Specificity of peptide selection by antigen-presenting cells homozygous or heterozygous for expression of class II MHC molecules: The lack of competition. *Proc. Natl. Acad. Sci. U.S.A. 100*, 5330-5335.

Tracey, B. M. and Shuker, D. E. (1997). Characterization of azo coupling adducts of benzenediazonium ions with aromatic amino acids in peptides and proteins. *Chem. Res. Toxicol. 10*, 1378-1386.

Walker, J. E. (1976) Lysine residue 199 of human serum albumin is modified by acetylsalicyclic acid. *FEBS Lett. 66*, 173-175.

Yasuzawa, T. and Tomer, K. B. (1997). Structural determination of the conjugate of human serum albumin with a mitomycin C derivative, KW-2149, by matrix-assisted laser desorption/ionization mass spectrometry. *Bioconjug. Chem. 8*, 391-399.

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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 naive 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 naive 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 (Roberts and Walter, 1998) 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.

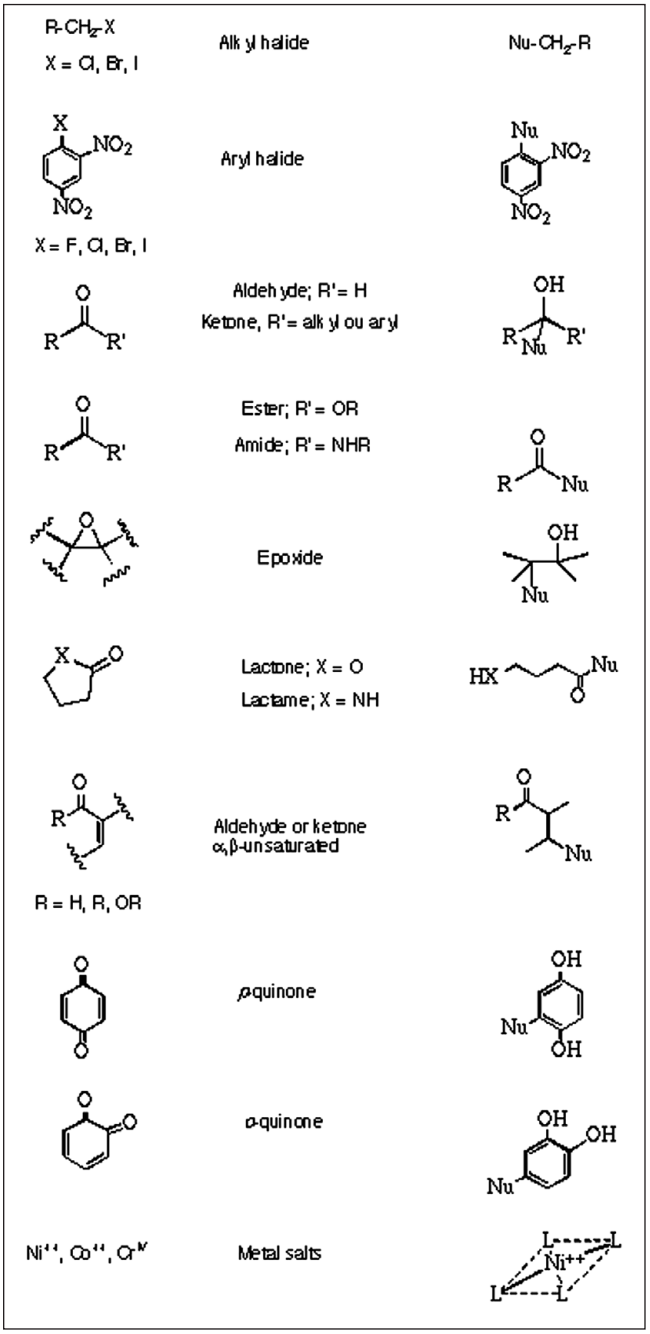
Main electrophilic chemical groups present in contact allergens

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 alkanesulphonates, allergenic methylating agents, mainly react with histidine and to a lesser extent with lysine, methionine, cysteine, and tyrosine (Lepoittevin and Benezra, 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.

Tab. 1: Main electrophilic groups seen in contact allergy with the products formed.



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

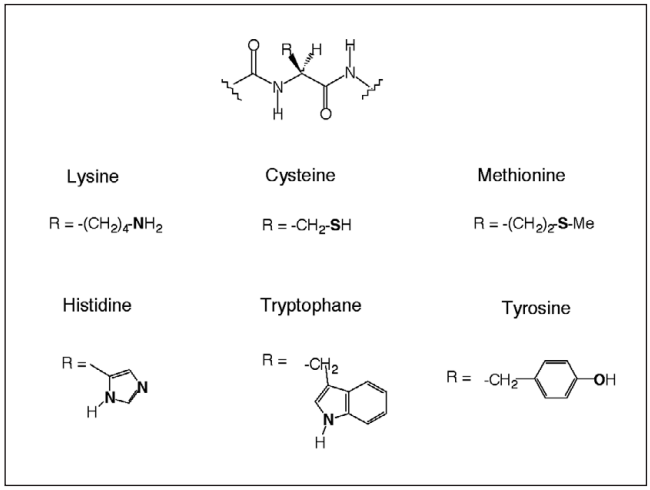


Fig. 1: Principal nucleophilic residues in proteins.

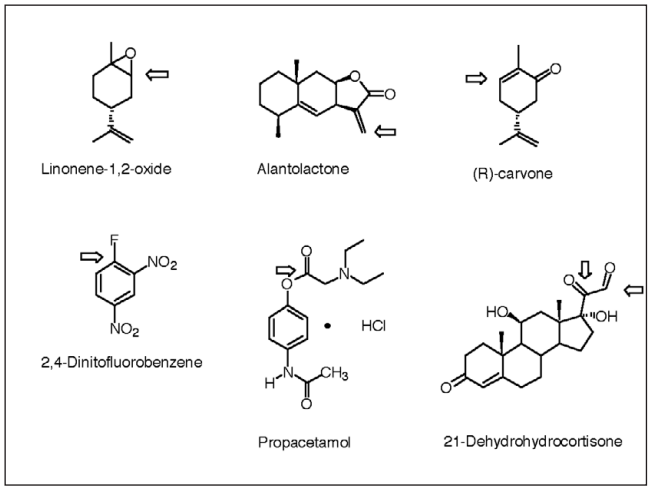


Fig. 2: Examples of sensitising molecules. The electrophilic centre is indicated by an arrow.

sensitising potential of two sultone derivatives, an alkenylsultone (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 proteins and in the density of these modifications. These differences, 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 haptenisation of the protein or proteins. It appears that the epitope density 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 selection 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 established, has been postulated to explain, for example, the allergenic potential of eugenol versus iso-eugenol (Barratt, 1992). More recently, studies indicating that radical reactions were important for haptens containing allylic hydroperoxide groups have been published (Lepoittevin and Karlberg, 1994; Giménez-Arnau, 2002).

Metabolism and pro-haptens

Far from being an inert tissue, the skin is the site of many metabolic processes, which can result in structural modification of the xenobiotics that penetrate it (Hotchkiss, 1998). These metabolic processes, primarily intended for the elimination of foreign molecules during detoxification, can, in certain cases, convert harmless molecules into derivatives with electrophilic, 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 monoamine oxidases, which convert amines to aldehydes, and peroxidases seem to play an important role in the metabolism of haptens. When activated by the production of hydrogen peroxide 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 hydrolyses 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, tulipalines 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 haptens, are referred to as pro-haptens (Landsteiner and Jacobs, 1936; Dupuis and Benezra, 1982) and play an important role in contact allergy because of their number and their highly reactive nature. The fact that the structure of the metabolised molecule can be far removed from the structure of the initial molecule can make allergologic investigations even more difficult.

Air oxidation and pre-haptens

Non-enzymatic processes, such as reaction with atmospheric oxygen or ultraviolet irradiation, can also induce changes in the chemical structure of molecules. Many terpenes spontaneously 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 monoterpene δ^3-carene (Hellerstrom et al., 1955). This is also the case for abietic acid, the main constituent of colophony, which is converted into the highly reactive substance hydroperoxide (Karlberg, 1988) by contact with air (fig. 4). Such an auto-oxidation mechanism has also been demonstrated for another monoterpene, *d*-limonene, found in citrus fruits. *d*-Limonene itself is not allergenic, but upon exposure to air hydroperoxides, epoxides, and ketones are formed that are strong allergens (Karlberg et al., 1994).

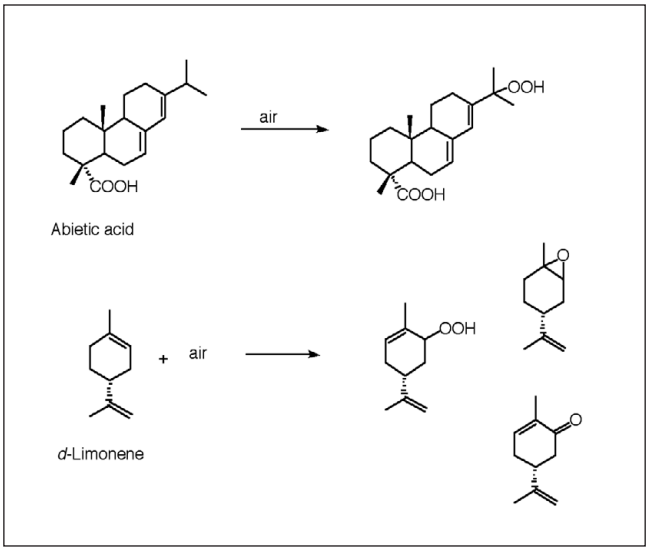


Fig. 3: Examples of pro-hapten metabolism.

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

Barratt, M. D. and Basketter, D. A. (1992). Possible origin of the skin sensitization potential of eugenol and related compounds. *Contact Dermatitis 27*, 98-104.

Bergmann, H. H., Beijersbergen, J. C. H., Overeem, J. C. and Sijpesteijn, A. K. (1967). Isolation and identification of α-methylene-γ-butyrolactone: A fungitoxic substance from tulips. *Rec. Trav. Chim. Pays-Bas 86*, 709-713.

Dupuis, G. (1979). Studies of poison ivy. In vitro lymphocytes transformation by urushiol protein conjugates. *Br. J. Dermatol. 101*, 617-624.

Dupuis, G. and Benezra, C. (eds.) (1982). *Allergic contact dermatitis to simple chemicals*. New York: Marcel Dekker.

Franot, C., Benezra, C. and Lepoittevin, J.-P. (1993). Synthesis and interaction studies of 13C labeled lactone derivatives with a model protein using ^{13}C NMR. *Biorg. Med. Chem. 1*, 389-397.

Frierson, M. R., Klopman, G. and Rosenkranz, H. S. (1986). Structure activity relationship among mutagens and carcinogens: A review. *Environ. Mutagen. 8*, 283-327.

Giménez-Arnau, E., Haberkorn, L., Grossi, L. and Lepoittevin, J.-P. (2002). Identification of alkyl radicals derived from an allergenic cyclic tertiary allylic hydroperoxide by combined use of radical trapping and ESR studies. *Tetrahedron 58*, 5535-5545.

Guengerich, F. P. and Liebler, D. C. (1985). Enzymatic activation of chemicals. CRC Crit. *Rev. Toxicol. 14*, 259-307.

Hellerstrom, S., Thyresson, N., Blohm, S.-G. and Widmark, G. (1955). On the nature of eczematogenic component of oxidized delta 3-carene. *J. Invest. Dermatol. 24*, 217-224.

Hosken, N. A., Shibuya, K., Heath, A. W. et al. (1995). The effect of antigen dose on $CD4^+$ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. *J. Exp. Med. 182*, 1579-1584.

Hotchkiss, S. A. M. (1998). Dermal metabolism. In M. S. Roberts and K. A. Walters (eds.), *Dermal absorption and toxicity assessment; Drugs and pharmaceutical sciences*, Vol. 91. New York: Marcel Dekker.

Karlberg, A.-T. (1988). Contact allergy to colophony. Chemical identification of allergens. Sensitization experiments and clinical experiments. *Acta Dermato-Venereol. 68 (Suppl. 139)*, 1-43.

Karlberg, A.-T., Shao, L. P., Nilsson, U. et al. (1994). Hydroperoxides in oxidized d-limonene identified as potent contact allergens. *Arch. Dermatol. Res. 286*, 97-103.

Landsteiner, K. and Jacobs, J. L. (1936). Studies on the sensitization of animals with simple chemicals. *J. Exp. Med. 64*, 625-639.

Lepoittevin, J.-P., Basketter, D. A., Goossens, A. and Karlberg A.-T. (eds.) (1998). *Allergic Contact Dermatitis: The molecular basis*, Berlin, Heidelberg, New York: Springer.

Lepoittevin, J.-P. and Benezra, C. (1992). ^{13}C enriched methyl alkanesulfonates: New lipophilic methylating agents for the identification of nucleophilic amino acids of proteins by NMR. *Tetrahedron Lett. 33*, 3875-3878.

Lepoittevin, J.-P. and Karlberg, A.-T. (1994). Interaction of allergenic hydroperoxides with proteins: A radical mechanism? *Chem. Res. Toxicol. 7*, 130-133.

Mansuy, D. (1985). Particular ability of cytochrome P450 to form reactive intermediates and metabolites. In G. Siest (ed.), *Drug metabolism: Molecular approaches and pharmacological implications*. New York: Pergamon.

Means, G. E. and Feeney, R. E. (1971). *Chemical modification of proteins*. San Francisco: Holden Day.

Meschkat, E., Barratt, M. D. and Lepoittevin, J.-P. (2001a). Studies of the chemical selectivity of hapten, reactivity, and skin sensitization potency. 1. Synthesis and studies on the reactivity toward model nucleophiles of the 13C-labeled skin sensitizers hex-1-ene- and hexane-1,3-sultones. *Chem. Res. Toxicol. 14* , 110-117.

Meschkat, E., Barratt, M. D. and Lepoittevin, J.-P. (2001b).

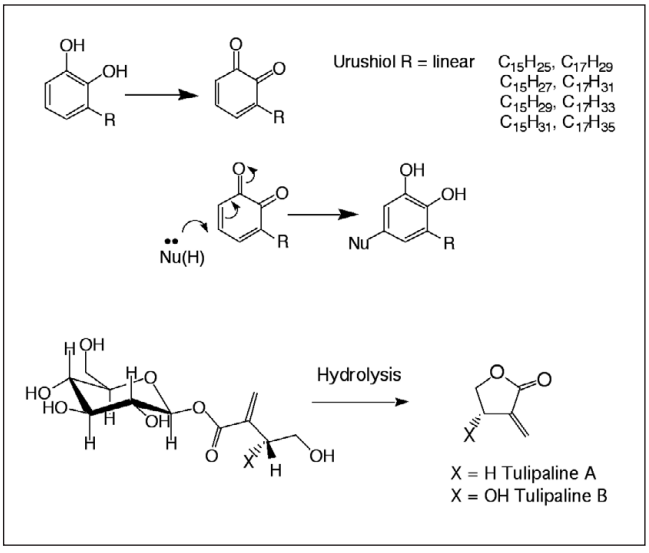


Fig. 4: Examples of chemical modification by reaction with air.

Studies of the chemical selectivity of hapten, reactivity, and skin sensitization potency. 2. NMR studies of the covalent binding of the 13C-labeled skin sensitizers 2-[13C]- and 3-[13C]hex-1-ene- and 3-[13C]hexane-1,3-sultones to human serum albumin. *Chem. Res. Toxicol. 14*, 118-126.

Reynolds, G. and Rodriguez, E. (1981). Prenylated hydroquinones: Contact allergens from trichomes of Phacelia minor and P. parryi. *Phytochemistry 20*, 1365-1366.

Roberts, M. S. and Walters, K. A. (eds.) (1998). *Dermal Absorption and Toxicity Assessment*, New York: Marcel Dekker.

Romagnoli, P., Labahrdt, A. M. and Sinigaglia, F. (1991). Selective interaction of nickel with an MHC bound peptide. *EMBO J. 10*, 1303-1306.

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