

Predictive Molecular and Genetic Toxicology

*Application to the Detection
of Sensitizing Potential of Xenobiotics*

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Introduction

It is essential to determine precisely the sensitizing potential of xenobiotics during immunotoxicological development studies. The prediction of idiosyncratic toxicity, especially contact hypersensitivity, is a major preoccupation in the chemical, pharmaceutical, and cosmetic industries. Such studies are required to avoid the onset of allergic reactions in users as much as several years after the initial release of a preparation, even if all legally required toxicological tests are negative.

It is currently agreed that the vast majority of undesirable effects of xenobiotics involve early, *in situ* molecular activation phenomena (Fig. 1). Although molecular toxicology has progressed in recent years, most observations have involved the hepatic toxicity of xenobiotics. However, there is no formal proof that these concepts can be extrapolated to the skin.

The aim of this article is to compare recent immunological data on contact hypersensitivity and our knowledge of the mechanisms underlying the bioactivation of xenobiotics in the skin. Using the example of contact allergy, we attempt to show how the analysis of very early molecular and/or genetic events in these two types of approaches can contribute to the predictive toxicology of idiosyncratic reactions to xenobiotics.

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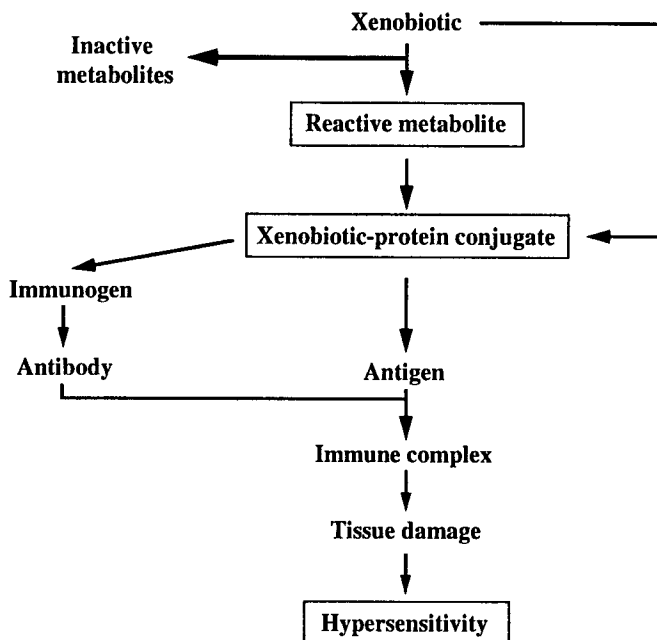


Fig. 1. Role of xenobiotic metabolism in the genesis of hypersensitivity reactions.

Contact Hypersensitivity to Xenobiotics

The pathophysiology of contact hypersensitivity (CHS), or allergic contact dermatitis in humans, is based on complex cellular and molecular interactions (1–3), which is not described here in detail. Certain principles required for the development of predictive *in vitro* tests are mentioned, however. Contact dermatitis is an inflammatory dermatosis observed in certain subjects after cutaneous contact (usually repetitive) with a compound behaving as a hapten and capable of penetrating into the skin, binding to dermal or epidermal cells, and provoking a delayed-type hypersensitivity reaction mediated by TH1 lymphocytes. It usually occurs after repeated cutaneous contact with environmental or occupational sensitizing agents. The different pathophysiological stages involve a clinically insignificant sensitization or induction phase after the initial contact, followed by an elicitation phase after reintroduction of the hapten, with the clinical onset of eczema (Fig. 2).

For a substance to cause contact allergy, it must first be capable of penetrating the skin. This phase may involve alterations of the stratum corneum owing to irritation phenomena, the dose applied, the repetitive nature of the contact, the lipophilic nature of the compound, and the vehicle used.

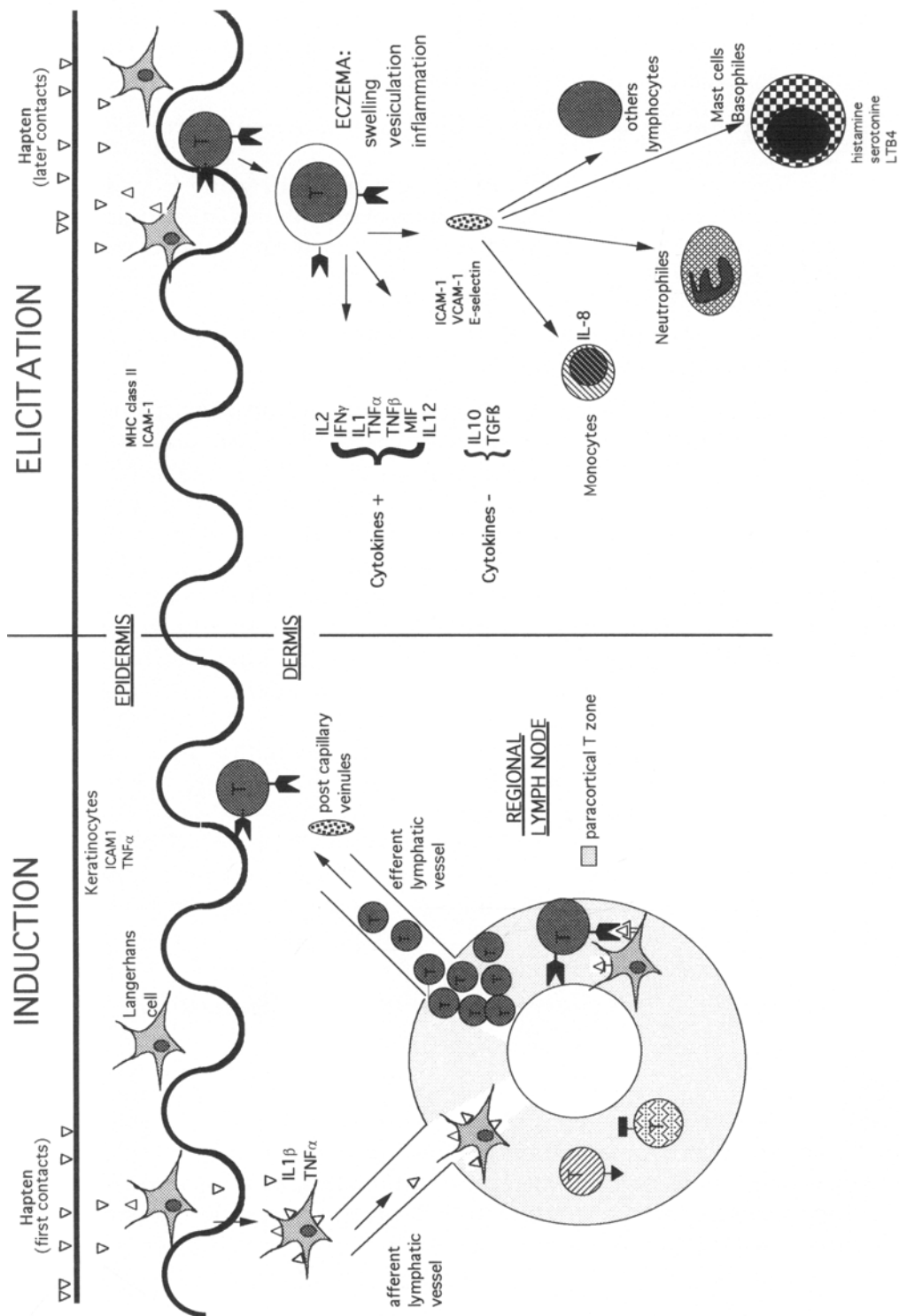


Fig. 2. Induction and elicitation phases in CHS.

Contact allergens share several characteristics. They are usually potential, incomplete allergens (haptens); they have a small molecular weight and, by definition, are not inherently immunogenic. The following categories may be distinguished:

1. Electrophilic haptens, which bind to nucleophile groups on proteins;
2. Compounds provoking oxidative stress and generating free radicals, which modify the structure of certain proteins; in both these cases covalent bonds are formed which transform the autologous (self) protein into a heterologous (non self) protein;
3. Lipophilic haptens, which bind directly to cell membrane lipids; and
4. Prohaptens, in which cutaneous or extracutaneous biotransformation probably plays an important role.

The induction phase occurs during topical application of a hapten in a nonsensitized subject. The hapten is processed by Langerhans' cells, which migrate to the draining lymph node and there present the antigen to naive T-cells. The antigen recognized by the T-lymphocyte may be an epitope of the major histocompatibility (MHC) antigen modified by the hapten, or a peptide present in the peptide-binding groove of the MHC molecule, also modified by the hapten (4,5). The hapten-specific T-cells then undergo clonal expansion and circulate throughout the body, infiltrating the peripheral tissues, like the skin. The elicitation phase occurs when the skin encounters the hapten again. Local memory TH1 cells are reactivated and release cytokines (IL-2 and IFN γ), thereby amplifying the reaction by direct activation of neighboring cells and recruitment of nonspecific mononuclear cells, and giving rise to the clinical manifestations of eczema.

Studies of identical twins (6–8) have suggested the existence of genetic factors, but environmental factors (especially repeated contacts with the allergen) probably play a determining role in the person-to-person variability of contact eczema.

Concepts Underlying Cutaneous Metabolization of Xenobiotics

Most undesirable effects of xenobiotics are owing to early molecular activation phenomena *in situ*. This involves the formation of unstable or reactive metabolites produced by the cells which represent important biological targets. These intermediate compounds are formed during xenobiotic activation (biotransformation) by several enzyme systems (mono-oxygenases, reductases, peroxidases, cyclo-oxygenases); in the liver, the most important enzyme system is the cytochrome P450 mono-oxygenase family.

Table 1
Cytochrome P450 in Human
and Rodent Skin and Liver

Family	Subfamily ^a	Liver ^b	Skin ^b	Refs.
I	IA (IA ₁ /IA ₂)	+	+	(12)
II	IIB (IIB ₁ /IIB ₂)	+	+	(13)
	IIC	+	?	
	IID	+	?	
	IIE (IIE ₁)	+	+	(14)
III	IIIA	+	+	(13,14)
IV	IVA	+	-	(15)

^aA new gene subfamily, named CYP IB1, has been recently identified in skin (16).

^b+, presence (in human and/or rodent skin or liver); -, absence; ?, not determined.

In qualitative terms, the skin has much the same enzymatic apparatus as the liver (9). Most oxidation, reduction, and hydrolysis reactions (phase I reactions) and conjugations (phase II reactions) have been demonstrated in the skin (10,11). The main differences are quantitative, as the skin only possesses 1–6% of the liver's metabolic capacities, depending on the species. Xenobiotic metabolism is nonetheless facilitated by the slow cutaneous blood circulation, which results in a relatively long contact time between xenobiotics and enzyme systems. These enzyme activities are mainly located in the epidermis, hair follicles, and sweat glands. The role of cytochromes P450 in cutaneous metabolism has mainly been studied because of the ubiquitous nature of these enzymes and their crucial role in xenobiotic metabolism. In mammalian tissues, 12 families have been identified, differing by their molecular weights, immunological reactivity, and substrate specificity. Isoenzymes involved in the metabolism of exogenous compounds, mainly belonging to families IA (IA1/IA2), IIB (IIB1/IIB2), IIE, and IIIA have been identified in rodent and human skin by means of biochemical, immunohistochemical, and molecular biology techniques (Table 1). Some of these activities can be induced or inhibited by topical treatment with substances active on the liver. The role of certain enzymes, especially isoenzyme IA1, in the metabolic activation of procarcinogens in the skin is well established. However, it remains poorly defined in the case of metabolic activation of prohaptens. By way of an example, the contradictory results of two recent studies are presented here.

In some cases, it is possible to exacerbate the sensitizing potential of prohaptens whose metabolization is cytochrome P450-dependent. In mice, monocrotaline is only sensitizing if the appropriate cytochrome P450 is stimulated by 3-methylcholanthrene (17). Inhibition of this P450

by piperonyl butoxide completely abrogates the CHS reaction to monocrotaline (induction and elicitation). In contrast, no contact sensitization is generated by other alkaloids such as atropine, ephedrine, or nicotine, probably because stimulation by 3-methylcholanthrene does not induce the appropriate cytochrome P450 isoenzymes.

Other teams have attempted to inhibit the enzyme systems thought to be involved. Eugenol and isoeugenol are contact allergens that must first be metabolized in the skin and transformed into the reactive hapten. Despite their similar chemical structures, they do not produce crossreactions; this has led to speculation that they could be activated by different mechanisms (18). Eugenol would be oxidized in the presence of cytochrome P450-IA, giving rise to free radicals derived from the OH group of the phenol function. With regard to isoeugenol, it would be demethylated then oxidized, giving rise to an orthoquinone. Scholes and coworkers (19) studied the effect of modulating the CHS reaction to these two haptens with clotrimazole, a known inhibitor of cytochrome P450-IA, expecting to observe an inhibition of the local lymph node assay (LLNA) response to eugenol and no change in the LLNA response to isoeugenol. However, pretreatment of mice with clotrimazole doubled the intensity of the inflammatory response to eugenol and quintupled the response to isoeugenol. These results are particularly interesting because they show that the situation is more complex than it appears. The authors suggested that cutaneous cytochrome P450-IA acts not by metabolizing eugenol into a more sensitizing product but rather by "detoxifying" the compound. These results, although confirming that clotrimazole indeed inhibits P450-IA, also suggest that it could stimulate other enzymes involved in the metabolism of eugenol and isoeugenol, thereby increasing their allergenicity.

Thus, although there is some evidence of the involvement of cytochromes P450 in the metabolic activation of prohaptens, current data on the cutaneous enzyme system and analytical conditions do not yet suggest that P450 play an important role in contact allergy. It should also be noted that other enzyme systems (flavine mono-oxygenases, peroxidases, etc.) present in the skin may play an important role in xenobiotic metabolism (20). Their part in the metabolic activation of prohaptens has not, to our knowledge, been investigated.

Fine regulatory mechanisms exist between the generation of toxic metabolites and detoxification processes of each substrate. These reactive metabolites can be formed in all tissues, as the relevant enzyme systems are ubiquitous. Schematically, it can be considered that two main mechanisms underlie the formation of these intermediate compounds (Fig. 3):

1. An oxidative pathway, with the formation of electrophilic compounds capable of binding covalently with biological macromol-

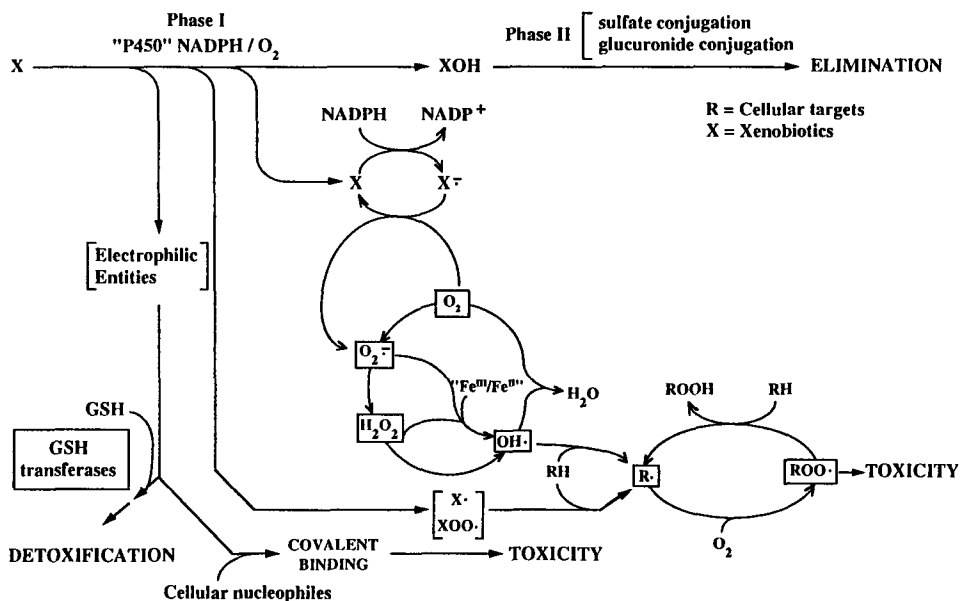


Fig. 3. Xenobiotic metabolism and cytotoxicity.

ecules. These irreversible bindings would thus be an early step in the onset of toxic effects.

2. A reductive pathway giving rise to intermediate compounds with an excess of electrons (free radicals, anionic radicals) which react with O₂ to produce reactive entities derived from the reduction of O₂ (e.g., superoxide anion).

The persistence of this oxidative stress could overwhelm antioxidant defense systems (superoxide dismutase, glutathion peroxidase, catalase, etc.), leading to the oxidation of biological macromolecules (lipids or proteins) and giving rise to toxic effects.

In addition, these two processes can also lead to glutathion depletion, which in turn accounts for a weakening of cell defenses. This is reminiscent of the fact that, with certain families of compounds (lactones, isothiocyanates), there is a correlation between the capacity for generating oxidative stress in the skin and the capacity for inducing CHS reactions (21).

However, the formation of these reactive metabolites is not alone sufficient to trigger adverse reactions. Very often it is a combination of several factors (dependent on the species and interindividual variations), which explains why some individuals react while others do not. This is what is meant by the expression "idiosyncratic toxicity." For example, internal factors such as the genetic variability of defense systems (polymorphism) and/or external factors (essentially induction

and inhibition of biotransformation and defense systems) can account for individual susceptibility to toxic intermediaries.

Relevance to Predictive Molecular and Genetic Toxicology

In the last two decades, studies of sensitizing potential have been based on one or several animal (guinea pig) tests. A more recent method developed in mice, the LLNA (22), is based on lymphocyte proliferation induced in the lymph node draining the site sensitized by the initial cutaneous application of a compound (induction phase), and not on inflammatory responses (observed after elicitation). Another advantage is that LLNA discriminates between contact allergens and aero-allergens, which induce TH1- and TH2-type responses, respectively. From this information, one can derive several strategies which might be applicable to predicting the sensitizing potential of xenobiotics, i.e., structure-activity studies according to chemical structure, and different approaches in vitro using simple or complex cell systems.

The QSAR Approach

Parallel with in vitro methods for detecting allergenic substances, quantitative methods for studying structure-activity relationships have been developed (QSAR). As hapten-protein interactions appear to be important in determining the sensitizing potential of the former, the relationship between the alkylating and sensitizing potential of various compounds has been extensively investigated in recent years (23,24). These authors defined a "relative alkylation index" (RAI), which takes into account three parameters: the amount of product applied to the skin, its lipophilicity, and its chemical reactivity. This approach, while innovative, remains only partial for two reasons. First, chemical reactivity is determined using an amine (butylamine), which is no doubt far removed from the characteristics of the proteins acting as targets for electrophilic species. Second, this system does not take into account metabolism.

In addition, certain databases systematically compile published clinical cases of contact allergy, as well as the results of experimental studies (25). Finally, computer-assisted decision systems are being developed for the prediction of sensitizing potential, a particularly exciting approach (26).

In Vitro Approaches

No in vitro tests for predicting sensitizing potential of molecules have so far been validated. Work in this field has involved in vitro studies of lymph node cells from animals sensitized in vivo, allergen bind-

ing capacity to proteins, exposure of cultured Langerhans' cells to allergens in vitro, or even studies of hapten binding capacity and induction of cell proliferation. Langerhans' cells (and other dendritic cells) can be used to generate primary proliferative responses by naive T-cells in vitro (27,28). Other in vitro approaches are being developed.

Subcellular Fractions

Various subcellular fractions (microsomes, mitochondria, plasma membranes) can be used in the early assessment of a compound's potential reactivity. Microsomal fractions in particular contain the main enzymes involved in xenobiotic metabolism. The capacity of a product to form electrophilic species or to generate oxidative stress can be evaluated biochemically in these models. Covalent binding of electrophilic species to nucleophiles (microsomal proteins) can be measured by means of radiometric methods, involving the radiolabeled compound. Various direct and indirect methods can also be used to assess the oxidative stress generated by reductive pathways. Studies using microsomes (interactions studies, metabolite identification, etc.) permit the enzymes involved to be phenotyped.

Monocellular Systems

During the induction phase of the CHS reaction, various cell types (keratinocytes, Langerhans' cells, and endothelial cells of dermal postcapillary venules) express a certain number of adhesion molecules and synthesize many cytokines (3,29). It has been suggested that certain of these early molecular signals might be specific for contact allergy reactions (3). These observations require confirmation, but nonetheless point to the value of work on an alternative predictive approach based on the measurement of events induced at the molecular level by sensitizing or irritant substances on easy-to-manipulate materials such as human keratinocyte or endothelial cell lines (30).

Another particularly promising tool is "purified forms," i.e., yeasts, bacteria, or mammalian cells transfected with a cDNA of human origin coding for a particular enzyme involved in xenobiotic metabolism (31). This might be useful for the fine analysis of mechanisms involved in the bioactivation of study compounds.

Multicellular Systems

Coculture systems combining T-lymphocytes, antigen-presenting cells, and a hapten (trinitrophenyl) can fully reproduce CHS reactions in vitro (27,32). Whereas the development of a purely in vitro test for all types of hapten appears unlikely in the near future, antigen-presenting cell lines could be used in the development of predictive tests for the primary response of naive T-cells in vitro, in the presence of molecular or cellular cofactors (which remain to be identified).

Another important phase is to determine the relative contribution of the different T-cell subpopulations in such systems. Results obtained in experimental (27,32) and human studies (33–35) stress the effector role of CD8+ T-cells in CHS. In addition, epidermal dendritic T-cells also appear to intervene in the early phase of CHS reactions (36). Finally, the molecular and cellular environment and the chosen culture conditions seem to be capable to orient the expansion of allergen-specific T-cell clones toward one phenotype or another, in terms of CD4 or CD8 membrane markers (35) or TH1/TH2 cytokine profiles (37).

With regard to the hapten, it must be tested in a chemical form activating T-cells. It is known that nickel binds to histidine residues and can bind to a peptide in the peptide-binding groove of MHC molecules (4), but hapten processing and presentation by antigen-presenting cells are generally poorly understood.

Human Reconstructed Skin Models

Cytochrome P450-dependent enzyme activities have been evidenced in the Episkin model (Imedex, Chaponost, France), a reconstructed human epidermis (38). After topical administration of various mono-oxygenase substrates, ethoxycoumarin and ethoxyresorufin activities were observed, showing the presence of the P450-IA cytochrome family. These two activities are strongly inducible by topical application of 3-methylcholanthrene. Hepatic P450 inducers (e.g., phenobarbital) have not shown stimulatory effects, reproducing the situation observed in the skin itself. Other results suggest that this model reproduces, *in vitro*, certain aspects of the cutaneous metabolic processes intervening *in vivo*. In addition, this model can be used to screen water-insoluble compounds and pharmaceutical/cosmetic preparations for their effect on cutaneous metabolism.

Other reconstructed human skin models such as Skin² possess cytochromes P450 1A1/A2, 2B1/B2 and 3A, as well as glutathion S-transferase (13). These models are under active development and will probably provide useful tools for the prediction of sensitizing potential.

Conclusion

The search for a link between cellular and molecular events involved in delayed-type CHS reactions and the early molecular activation of xenobiotics is a new field of research. It should largely contribute to the debate on the best way forward for predictive toxicology in general.

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