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Review

Chemical reactivity measurements: Potential for characterization of respiratory chemical allergens

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ABSTRACT

Allergic diseases of the skin and respiratory tract resulting from exposure to low molecular weight chemicals remain important issues for consumer product development and occupational/environmental health. Widespread opportunities for exposure to chemical allergens require that there are available effective methods for hazard identification and risk assessment. In the search for new tools for hazard identification/characterization there has been interest in developing alternative methods that will reduce, refine or replace the need for animals. One approach that shows promise is based on the measurement of the peptide reactivity of chemicals; the potential to form stable associations with protein/peptide being a key requirement for the induction of sensitization. Recent investigations using these systems have focused primarily on skin sensitizing chemicals. However, there is interest in the possibility of exploiting these same experimental approaches to distinguish between different forms of chemical allergens – as individual materials are primarily associated with one or the other form of sensitization in humans. These investigations may also provide insight into why chemical sensitizers can differ in the form of allergic disease they will preferentially induce. These opportunities are surveyed here against a background of the immunobiology of allergic sensitization and current state-of-the-art approaches to measurement of peptide/protein reactivity.

Contents

Abbreviations: ACD, allergic contact dermatitis; AHCP, ammonium hexachloroplatinate; APC, antigen presenting cells; Da, Dalton; DAD, diode array detector; DC, dendritic cell; DNCB, 2,4-dinitrochlorobenzene; DNFB, 2,4-dinitrofluorobenzene; DTT, d,l-dithiothreitol; EC50, effective concentration 50; EU, European Union; FIA, flow-injection analysis; FITC, fluorescein isothiocyanate; FR, factor of reactivity; GSH, glutathione; HHPA, hexahydrophthalic anhydride; HSA, human serum albumin; HDI, hexamethylene diisocyanate; HPLC, high-performance liquid chromatography; HSAB, hard and soft acid base theory; IFN-γ, interferon-gamma; Ig, immunoglobulin; IL, interleukin; LC, Langerhans' cell; LLNA, local lymph node assay; MDI, diphenylmethane diisocyanate; MHC, major histocompatibility complex; MS, mass spectroscopy; NIH, National Institutes of Health; NMR, nuclear magnetic resonance; OECD, Organization for Economic Co-Operation and Development; PA, phthalic anhydride; QSAR, quantitative structure activity relationship; REACH, registration, evaluation, authorization and restriction of chemicals; RIFM, Research Institute for Fragrance Materials; TDI, toluene diisocyanate; TMA, trimellitic anhydride; TNF, tumor necrosis factor; TNP, trinitrophenyl; UV, ultra violet.

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1. Introduction

Allergic hypersensitivity can be defined as an inappropriate immune response to a normally innocuous antigen resulting in tissue injury and adverse health effects (Janeway et al., 1997). Many classes of materials encountered in the environment may provoke such an immune response. Broadly, these materials may be divided into two categories: high molecular weight compounds, greater than 1000 daltons (Da) and low molecular weight chemicals, less than 1000 Da. High molecular weight compounds are able to interact with the immune system directly to provoke an immune response; these include proteins encountered in the environment or occupationally; such as pollen, house dust mite excreta, animal dander and enzymes (Chapman et al., 2007). In contrast, chemicals of low molecular weight are too small to be recognized by the immune system directly and act as haptens, which must first react directly or indirectly with a protein in order to provoke an immune response. Of particular importance in the contexts of occupational health, predictive toxicology and ensuring the safety of manufactured products is the ability of some chemicals to cause allergic sensitization of the skin and respiratory tract.

Skin sensitization resulting in allergic contact dermatitis (ACD) is by far the most frequent manifestation of immunotoxicity in humans and a common occupational disease. Several hundred chemicals have been implicated as skin sensitizers based on human experience and many more have been identified as potential allergens based on experimental models. Extensive databases have been published including over 200 representative examples of skin sensitizing chemicals (Gerberick et al., 2005; Kern et al., 2010). There are a number of experimental models available to assess the potential for a chemical to act as a skin sensitizer. Historically, guinea pigs were the species most commonly used for skin sensitization hazard identification (Buehler, 1965; Magnusson and Kligman, 1969). In these assays, the potential of a material to act as a skin sensitizer is based on visual scoring of skin responses (erythema/edema) following challenge of previously sensitized animals. More recently, the murine local lymph node assay (LLNA) has been increasingly used for hazard assessment (Kimber and Basketter, 1992; Kimber et al., 1994, 2002a). In the LLNA, the sensitizing potential of a chemical is assessed quantitatively by measurement of cell proliferation in the lymph nodes draining the site of topical exposure to the test material. The LLNA has been validated as an alternative to the more traditional guinea pig models for the identification of potential skin sensitizers (Gerberick et al., 2000; National Institutes of Health [NIH], 1999; Organization for Economic Co-Operation and Development [OECD], 2002). In addition to its application as a method for hazard identification, the LLNA provides a basis for the objective and quantitative measurement of relative skin sensitizing potency (Basketter et al., 2000). In the context of human health risk assessments, the ability to determine potency is of particular importance since the skin sensitizing potency of chemical allergens can vary by several (and probably up to 5) orders of magnitude.

In contrast to skin sensitizers, there are far fewer chemicals that are known to cause respiratory allergy. Though less numerous, respiratory sensitizers are of concern because respiratory allergy is commonly associated with high levels of morbidity, and occasionally mortality, and has significant socio-economic consequences (Bernstein et al., 2006; Kimber and Dearman, 1997). In the context of this review sensitization of the respiratory tract refers to immunologic priming resulting from the initiation of an immune response by a chemical allergen. The chemicals that can cause an asthmatic response via these immunologic mechanisms are considered to be true 'respiratory allergens'. This is distinct from similar physiological changes that can occur via non-immunological mechanisms, in which case the offending chemicals and agents have been termed 'asthmagens' (Kimber et al., 2007).

Published reviews, that have sought to identify potential chemical respiratory allergens based on clinical evidence, have reported numbers in the range of 40–80 materials (Graham et al., 1997). Chemicals generally agreed to cause allergic sensitization of the respiratory tract resulting in occupational asthma include: diisocyanates (such as diphenylmethane diisocyanate [MDI], hexamethylene diisocyanate [HDI] and toluene diisocyanate [TDI]); acid anhydrides (such as trimellitic anhydride [TMA] and phthalic anhydride [PA]); metals (such as certain platinum salts), pharmaceuticals and their intermediates (such as penicillin and phenylglycine acid chloride) and other industrial agents that have applications in painting, dye making, plastics and electronics manufacturing (ECETOC, 1999; Graham et al., 1997; Kimber and Dearman, 1997). Discrepancies between the numbers of allergens identified reflect the individual authors' criteria for classifying a chemical as a respiratory sensitizer. These criteria often ascribe the highest weighting to clinical reports of varying quality to identify potential allergens and may therefore include asthmagens as well as respiratory sensitizers. The default to clinical indications for identification of potentially hazardous materials has been driven by the fact that there are no validated or generally accepted models to identify respiratory sensitization hazards (reviewed by Kimber et al. (2007)). The lack of an agreed methodology is the direct result of controversy and lack of understanding of the underlying mechanisms leading to sensitization of the respiratory tract.

Given the extent of human exposure to chemicals that may have the potential to cause allergic disease, there is a need to identify hazards and to conduct accurate risk assessments to protect health. The requirement for a high degree of confidence with regards to the accuracy of risk assessments is an important consideration. The aim is to safeguard public health, while not overly restricting the use of materials that have important potential benefits. The quality of assessments is reliant on the tools, methodologies and information available. Current risk assessment practices rely almost exclusively on *in vivo* models. There is significant social, scientific and economic pressure to replace animal testing where possible. The most pressing example is that of the European Union (EU) ban on *in vivo* testing of cosmetic and toiletry ingredients, which came into general force in 2009 (EU Directive 2003/15/EC). Registration, evaluation, authorization and restriction of chemicals (REACH) legislation has also mandated that *in vivo* testing be conducted only when appropriate alternatives are not available (EU, 2002). Irrespective of legislative pressures, there is a significant public desire, and indeed an ethical responsibility, to reduce, refine or replace (3Rs) animal testing wherever this is possible. In recent years, progress has been made in the design of *in vitro* tools for the identification of skin sensitizers. One important development has been the application of chemical reactivity measurements to the identification of skin sensitizing chemicals (Gerberick et al., 2004, 2008). Given their apparent utility with respect to the identification of skin sensitizers, there has been

interest in exploring these methodologies for the identification of chemical respiratory sensitizers and for probing mechanistic differences that may lead to either form of chemical allergy. From a risk assessment and risk management perspective, it is important to distinguish between the two qualities of allergic response. This review will consider the chemical and immunobiological aspects of skin and respiratory chemical allergens that may provide information to assist in their identification, characterization and classification.

2. Immunobiology of sensitization

The nature of the cellular and molecular events that result in the acquisition of skin sensitization to chemicals is relatively well characterized, although our knowledge is far from complete. This process has been reviewed extensively (Dearman and Kimber, 2003; Kimber et al., 2002b; Smith and Hotchkiss, 2001). In contrast, the events leading to sensitization of the respiratory tract by chemicals are not clearly understood and remain controversial. Recent workshops and conferences have been held with the goal of developing consensus on the basic etiology leading to chemical respiratory sensitization and identification of future research paths (Kimber et al., 2007; Isola et al., 2008).

Although it is not appropriate to present here in full the immunobiology of skin and respiratory sensitization, the brief review below serves to highlight the key areas that may provide starting points for the development of novel methodologies and approaches to assess hazard and potency. Although skin and respiratory sensitization share some general similarities, there are clear mechanistic differences that become apparent as the immune response develops. In fact, it is uncommon for a chemical allergen to be implicated as causing both skin and respiratory tract sensitization in humans. It is perhaps best to consider first the better defined process of skin sensitization, and then what is known of the mechanism of respiratory sensitization to chemicals and how this differs from skin sensitization.

2.1. Skin sensitization

Within a clinical setting, skin sensitization typically manifests with a patient complaining of symptoms collectively defined as "ACD". Such individuals present with varying degrees of erythema, edema and vesiculation on the skin following contact with an allergen to which they have been sensitized previously (Rycroft et al., 2001). The acquisition of skin sensitization resulting in ACD results from a complex cascade of chemical and biological events. There are two distinct phases in the process. First is the induction phase, in which the immune system is primed to recognize and react to an antigen. Second is the elicitation phase, the clinical manifestations of ACD, in which a previously primed immune system reacts more aggressively on subsequent exposure to the same material.

In order for a chemical to act as a skin sensitizer, it must first cross the stratum corneum and access the viable epidermis and dermis. Penetration must occur at a rate that ensures enough chemical is available to react with proteins in the epidermis and produce sufficient quantities of conjugate (Basketter et al., 2007). Protein reactivity is an important step in the process as the size of the typical chemical allergen (<1000 Da) is too small to be recognized directly by the immune system. Such chemicals therefore behave as haptens (Dupuis and Benezra, 1982). The chemical may be inherently reactive, or may acquire reactivity via metabolic activation or autoxidation (Lepoittevin et al., 1998; Smith and Hotchkiss, 2001). Along with protein reactivity, it is believed that the chemical must cause a degree of skin trauma that results in the up-regulation of epidermal cytokines (such as tumor necrosis factor [TNF]-α) and interleukins [IL] (such as IL-1β, IL-18) (Cumberbatch et al., 2003). This so called "danger signal" is essential in order to support an immune response.

Within the epidermis and dermis reside dendritic cells (DC) that are responsible for the recognition, internalization and processing of allergens. Langerhans cells (LC), a subpopulation of bone marrow derived epidermal DC, have been classically considered the sole antigen presenting cells responsible for the initiation of skin sensitization. More recently, it has been shown that various populations of DC exist – particularly within the dermis – that play pivotal roles in generating and regulating immune responses (reviewed in Guilliams et al., 2010; Zaba et al., 2009). Work in mice lacking LC, but with functional dermal DC, has called into question the classic paradigm and demonstrated that there are circumstances in which the development of skin sensitization can proceed in the absence of LC (Kaplan et al., 2005; Kissenpfennig and Malissen, 2006; Noordegraaf et al., in press; Poulin et al., 2007; Wang et al., 2008). There is a great deal of uncertainty about the role each cell population plays relative to the other. The interpretation thus far is that LC and dermal DC have a complementary, and possibly mutually redundant, role in antigen presentation.

Irrespective of their relative roles, LC and dermal DC initiate the process of adaptive immunity upon encountering the hapten–protein complex. The migration of LC is perhaps the most studied and serves as an instructive example of skin resident DC trafficking. LC are stimulated to migrate from the epidermis via draining lymphatics to the regional lymph nodes following internalization and processing of antigen (Cresswell, 2005; Cumberbatch et al., 2003). En route, the LC under go a process of maturation during which the cells down-regulate antigen-processing capability and direct the cellular machinery towards antigen presentation and immunostimulation (Banchereau and Steinman, 1998). Langerhans cells arrive in the lymph nodes as fully capable antigen-presenting cells and the processed antigen is presented to responsive T lymphocytes (Guermonprez et al., 2002). This antigen presentation takes place in the context of major histocompatibility complex (MHC) class II molecules (Bromley et al., 2001). The T cells and LC associate together in the paracortical region of the node and presentation occurs following development of an immunological synapse (Grakoui et al., 1999). The ultimate result is the selective clonal expansion of responsive T cells which culminates in the release into the circulation of allergen-specific reactive lymphocytes. This represents the completion of the induction phase and signals the acquisition of cellular immunological immunity and memory.

The sensitized individual is now primed to mount an accelerated and more aggressive response upon subsequent exposure to the same allergen. During this elicitation phase, the same process of skin penetration and protein reactivity is necessary (Basketter et al., 2007). The antigen complex is then internalized, processed and presented to circulating T cells that have migrated into the dermis. The antigen presenting cells (APC) may be LC or dermal DC; however at this stage other cells such as keratinocytes or macrophages may act as APC to memory T cells (derived from previous clonal expansion during the induction phase) (Nickoloff and Turka, 1994). Migratory cells, such as LC, dermal DC and macrophages, may also present antigen in the lymph nodes. In the skin, antigen recognition by memory T cells results in a cascade of proinflammatory cytokine signaling and expression within the epidermis and dermis. This chemical milieu stimulates the infiltration of monocytes, macrophages and T cells which results in the clinical expression of ACD.

2.2. Sensitization of the respiratory tract

The clinical picture of respiratory hypersensitivity is most often associated with the symptoms of asthma and rhinitis. The

responses can occur soon (within minutes) after challenge of a previously sensitized individual and symptoms can include bronchoconstriction, wheezing, breathlessness, rhinitis and conjunctivitis. These reactions can in the worst of cases develop into potentially life threatening anaphylactic shock (Bernstein et al., 2006).

In comparison to skin sensitization to chemicals, the biologic events leading to sensitization of the respiratory tract are less clear. While it is known that respiratory sensitization to protein allergens is associated with, and dependent upon, IgE antibody, there is no such consensus with respect to chemical respiratory allergy (Kimber and Dearman, 2002). Although there is evidence in some individuals for the production of specific IgE antibody to the majority of chemicals confirmed as respiratory allergens, there are symptomatic individuals with diagnosed occupational asthma who apparently lack detectable IgE. The latter is true especially with respect to the diisocyanates where a significant number of symptomatic patients are reported to lack measurable serum IgE antibody (Bernstein et al., 2002; Cartier et al., 1989). This may signal that other immunological mechanisms promote sensitization of the respiratory tract to some chemicals. However, it remains possible that the lack of association with IgE antibody is due to technical difficulties in measuring the antibody and/or a reflection of the fact that serological studies have frequently been conducted some time after the last exposure to the inducing chemical allergen (Park et al., 2001; Tee et al., 1998). It may also be the case that the reagents used to detect antibody are inadequate under some circumstances (Karol, 2002). Nevertheless, the possible contributions of IgE-independent immunological processes cannot be discounted.

In both skin and respiratory sensitization T cell activation plays a critical role. However, each process appears to exhibit a preferential activation of different subpopulations of T helper (Th) cells (Dearman et al., 2003). Two functional subpopulations of Th cells, and the balance of their resultant cytokine expression, play pivotal roles in determining the quality of immune response that develops to an allergen. The two subpopulations, Th1 and Th2 phenotypes, develop from the same precursor cells (Th0) (Bendelac and Schwartz, 1991). In skin sensitization there is preferential activation of Th1 type cells which promote cell mediated immunity and are characterized by production of IL-2, interferon-gamma (IFN-γ) and tumor necrosis factor-β (TNF-β). Respiratory sensitization to chemicals leads to a predominately Th2 type response promoting IgE antibody responses and is associated with IL-4, IL-5, IL-6, IL-10 and IL-13 secretion. There is general agreement that irrespective of an absolute requirement for IgE antibody, T helper 2 (Th2)-type immune responses favour allergic sensitization of the respiratory tract (Kimber and Dearman, 2005).

In the case of respiratory sensitization, inhalation exposure to chemicals would seem to be the most appropriate route for the induction of sensitization. However, there is evidence to suggest that under some circumstances skin exposure may be effective for acquisition of respiratory sensitization (Blaikie et al., 1995; Botham et al., 1988; Pauluhn et al., 2005). Interestingly, the LLNA and guinea pig assays which are used for identifying skin sensitization hazards also result in positive responses when conducted with respiratory allergens (Kimber et al., 2007). Although these observations have been predominately in experimental animals, there are reports in humans of effective respiratory sensitization occurring through significant skin contact (Beck and Leung, 2000; Liu et al., 2000).

3. The chemistry of sensitization to chemical allergens

The correlation between skin sensitization potential and protein reactivity is well-established and has been appreciated for some time (Dupuis and Benezra, 1982; Lepoittevin et al., 1998). While the bulk of the theoretical and mechanistic foundations of this relationship are based on experience with skin sensitizers, there is evidence to suggest that chemical reactivity also plays an essential role in the development of respiratory sensitization to chemicals.

3.1. Reaction mechanisms

The interaction of chemical allergens with biological macromolecules can proceed by a variety of mechanisms and result in bond formations of various strengths (Lepoittevin et al., 1998). Weak interactions characterized by hydrophobic, dipolar and ionic bonds produce conjugates of low stability and take place at relatively low energy levels (<50 J/mol). These interactions are of biological importance affecting, among other parameters, bioavailability, receptor interactions and constraining enzyme activity (Brain and Chilcott, 2008; Voiculetz et al., 1993). However, strong covalent bonds, characterized by energies in the range of 200–450 kJ/mol, are of most significance for the formation of immunogenic conjugates. The electronic interactions relevant for sensitization can be modeled on the hard and soft acid base theory (HSAB) (Pearson, 1963). Based on HSAB theory, hard electrophiles (electron-poor) have an increased reactivity to hard nucleophiles (electron-rich) with the reaction taking place on low-energy orbitals of low polarizability. Conversely, soft electrophiles have an increased reactivity to soft nucleophiles with the reaction taking place on higher-energy orbitals of high polarizability. There are a number of potential reaction mechanisms available and, with respect to skin sensitization, three categories are generally agreed to be the most relevant: nucleophilic substitution on a saturated center, nucleophilic substitution on an unsaturated center and nucleophilic addition reactions (Fig. 1). While these fundamental classes of substitution and addition reactions form the basis of reactivity to skin sensitizing chemicals, the underlying reaction mechanisms are more complex. Evaluation of published datasets of skin sensitizers and the development of (quantitative) structure activity relationship [(Q)SAR] models for skins sensitizing chemicals has shown that these reaction classes can be further divided and the reactive chemicals most often described as either – Michael acceptors, S_NAr electrophiles, S_N2 electrophiles, Schiff base formers or acylating agents (Aptula et al., 2005; Aptula and Roberts, 2006).

The reaction mechanisms associated with skin sensitizing chemicals are likely to have relevance for the interaction of respiratory sensitizers with proteins. Statistical analysis of the functional groups within common respiratory sensitizers and asthmagens has shown a strong correlation with the presence of reactive groups such as isocyanates, amines, acid anhydrides and carbonyls (Jarvis et al., 2005). A review of the electrophilic reaction chemistries of selected respiratory allergens showed that similar pathways and mechanisms exist to those observed for skin sensitizers (Enoch et al., 2009). There have been several attempts at developing (Q)SAR models for the prediction of respiratory sensitization and asthmagen hazards (Cunningham et al., 2005; Jarvis et al., 2005; Karol et al., 1996). Interestingly, these efforts have often identified a strong correlation between the presence of multiple reactive functional groups and the ability to induce respiratory allergy. With respect to skin sensitizing chemicals, the presence or absence of multiple reactive functional groups has not been considered to be of significance. The mechanistic interpretation has been that cross-linking, within or between proteins, is an important characteristic of chemical respiratory allergens. However, there are respiratory sensitizers for which a cross-linking mechanism may not be the most relevant reaction. For example, Enoch et al. (2009) identified PA as a material which can under go an irreversible acylation reaction. Under these conditions, despite the

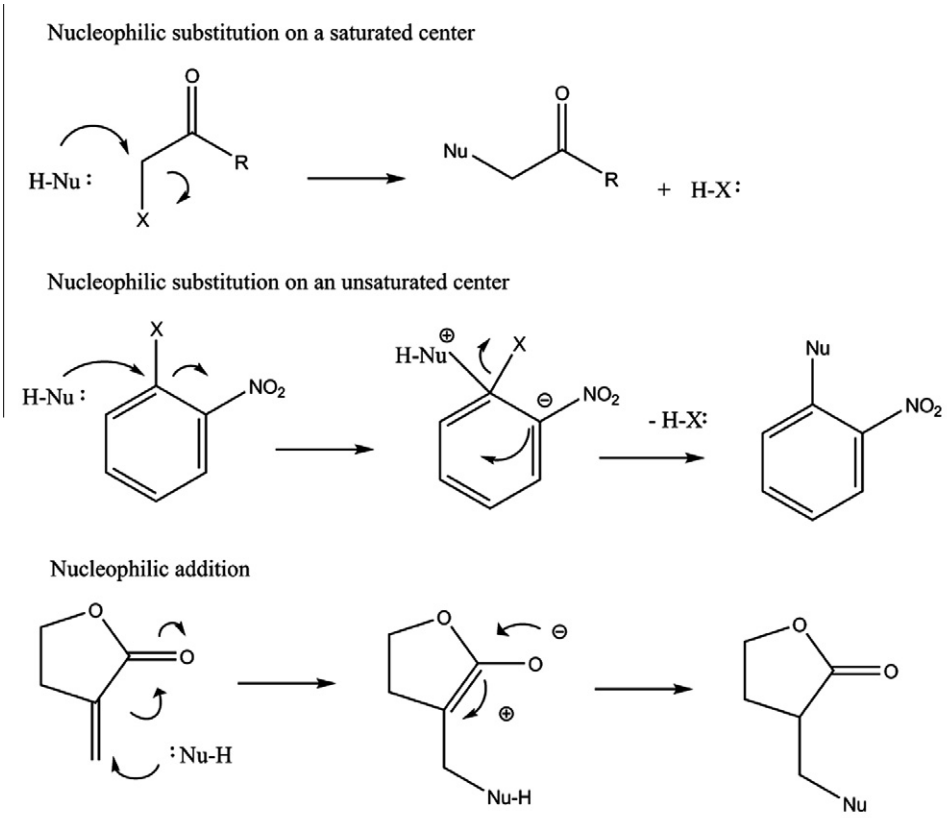

Fig. 1. Examples of relevant reaction pathways for covalent bonding of chemical allergens with proteins and peptides. The interaction of chemical allergens with biological macromolecules can proceed by a variety of mechanisms; however those that result in strong covalent bonding are of greatest significance for the acquisition of sensitization. For skin sensitization, three reaction pathways are generally agreed to be the most relevant: nucleophilic substitution on a saturated center, nucleophilic substitution on an unsaturated center and nucleophilic addition reactions. Abbreviations: Nu, nucleophile.

apparent presence of multiple functional groups, the authors suggest that the anhydride function within PA may act as a single monofunctional center. Enoch et al. (2009) further introduced the concept of a 'reactivity threshold' which takes into account both electrophilic and cross-linking ability. The theory is that a highly electrophilic allergen will compensate for a lack of cross-linking, surpass a theoretical threshold, and act as a respiratory sensitizer. Modeling to date has provided insight into key reaction mechanisms, the continued improvement in QSAR models will inform both hazard assessment and the identification of relevant reaction mechanisms (see Patlewicz and Worth, 2008; Seed et al., 2008).

3.2. Peptide and protein reactivity – skin sensitization

With respect to skin sensitizers, the most often cited and earliest historical reference regarding reactivity and sensitization potential is the work of Landsteiner and Jacobs (1936). In this landmark paper, the authors observed that skin sensitization in guinea pigs to a series of nitro- and chloro- substituted benzenes was associated with the reactivity of the materials to aniline, and with a generally increased rate constant for decomposition by reaction with sodium methylate. Subsequent observations by numerous researchers have established the 'electrophilic' theory of skin sensitization (Lepoittevin et al., 1998; Roberts et al., 2007). This view of sensitization is based on chemical allergens acting as electrophiles and reacting with nucleophilic amino acid residues within proteins in a stable, covalent manner. The non-self, hapten–protein complex generated is then sufficient to elicit an immune response.

Inherent reactivity of the parent compound is not the only determinant of the ability of a chemical to act as a hapten, nor is it a guarantee that a chemical will be recognized as foreign. Chemicals requiring transformation to reactive species via non-enzymatic processes have been termed pre-haptens; and those requiring enzymatic processes have been called pro-haptens (Smith and Hotchkiss, 2001). A non-reactive chemical may be transformed into a hapten by auto-oxidation. For example, it has been demonstrated that highly pure samples of the fragrance materials limonene and linalool are not considered to be skin sensitizers. However, products formed by these materials following exposure to air are known to have allergenic potential (Karlberg et al., 1992). It should be noted that exposure to air may also result in a decrease in sensitizing activity of some compounds. For example, autoxidation of cinnamic aldehyde by air exposure was observed to result in a decreased sensitization potential, presumably due to degradation or steric hindrance of the aldehyde functionality on the product (Research Institute for Fragrance Materials [RIFM], 2003).

Dupuis and Benezra (1982) hypothesized more than 25 years ago that metabolic activation by cutaneous enzymes may play a role in skin sensitization to chemicals. Recent work has demonstrated that incubation of the non-sensitizing/weakly sensitizing fragrance material geraniol with skin like cytochrome P450 enzymes (a family of ubiquitous skin and liver metabolizing enzymes of the mixed function oxidase system) produced sensitizing epoxide intermediates and degradation products (Hagvall et al., 2008). Similar mechanisms may also act to render a reactive chemical less potent or non-sensitizing. For example, cinnamic aldehyde has been shown to be reduced by alcohol dehydrogenase in human

skin to a non-sensitizing metabolite, cinnamic acid (Smith et al., 2000).

Protein and peptide modification is generally agreed to be a prerequisite for the acquisition of skin sensitization. This requirement was demonstrated in a series of experiments showing that trinitrophenyl (TNP) sensitization in mice was associated with TNP bound to lysine within peptides and the subsequent complex anchoring to the grooves of MHC molecules (Kohler et al., 1995; reviewed by Weltzien et al. (1996)). However, identifying more generally the specific proteins and/or binding sites that are immunologically relevant has remained a significant challenge. Work by Elahi et al. (2004) identified a wide spectrum of modified proteins following *in vitro* incubation of cinnamic aldehyde with rat and human skin homogenates. Other researchers have demonstrated, with both allergens and more general reactive electrophiles, that there may be protein targets that are selectively modified (Dennehy et al., 2006; Hopkins et al., 2005; Shin et al., 2007). In fact, experiments conducted with smaller peptides have revealed that the selective modification of amino acid residues by chemical allergens may explain observed biological responses. Meschkat et al. (2001a,b) showed that the difference in skin sensitizing potency between alkylsultone and alkenylsultone is best explained by the formers preference for binding lysine. Similar observations have been made for the Kathon CG components – 5-chloro-2-methylisothiazol-3-one and 2-methylisothiazol-3-one (Alvarez-Sanchez et al., 2004a,b). Presently, there is no definitive evidence identifying whether promiscuous or more specific protein modifications are the norm within intact human skin. If it is the case that promiscuous binding is more prevalent, the specificity of skin allergy to chemicals would suggest that, at very least, a subset of modified proteins could serve as unique immunologic determinants.

3.3. Peptide and protein reactivity – respiratory allergens

As mentioned previously, mechanistic understanding of the relationship between allergenicity and electrophilic reactivity is based predominately on studies with skin sensitizers. Evidence to date suggests that the acquisition of respiratory sensitization to chemicals is dependent upon similar covalent interactions with proteins.

The concept of a carrier protein has long been a feature of clinical and experimental studies with chemical respiratory allergens. The free chemical is typically not recognized within a given assay until it is bound to a protein, often albumin. In the case of isocyanates, there is a variety of published methods for generating albumin conjugates for use in clinical immunologic assays (Karol et al., 1978; Scheel et al., 1964; Tse and Pesce, 1979). Such conjugated proteins are known to have conserved binding sites and elicit highly specific humoral responses. Wisenewski et al. (2004) demonstrated that human serum albumin (HSA) conjugated with HDI exhibited two predominate and conserved binding sites – Lys^{414} and Cys^{247}. The authors conjectured, based on a comparative serological study which included HDI conjugated bovine serum albumin (lacking the Lys^{414} conjugation site of HSA), that antibody specificity may in part be explained by the biochemical specificity of the HDI reaction with albumin.

In situ derived protein conjugates have been observed in both humans and animal models following inhalation exposure to chemical respiratory allergens. For example, protein conjugated hexahydrophthalic anhydride (HHPA) has been found in nasal lavage fluid of exposed workers, and in guinea pig models of respiratory sensitization (Johannesson et al., 2004). HHPA conjugated to serum albumin within plasma and to hemoglobin has also been reported (Lindh and Jönsson, 1998; Rosqvist et al., 2000). Several isocyanates have been observed conjugated to lung epithelial cell proteins and bronchial lavage fluid proteins from exposed human workers, guinea pigs and the mouse (Jin et al., 1993; Karol and Jin, 1997; Kennedy and Brown, 1992; Lange et al., 1999; Wisnewski et al., 1999). The presence of these conjugated proteins does not, in itself, confirm the antigenic form of the allergens. It does demonstrate, however, that under physiological conditions, respiratory allergens may readily react with a variety of proteins.

4. Approaches for the identification of allergens based on chemical reactivity measurements

There has been significant progress in the development of *in vitro* assays for the predictive identification of skin sensitizing chemicals based on chemical reactivity measurements (reviewed extensively by Gerberick et al. (2008)). There are some reports, although fewer in number, of attempts to identify respiratory sensitizers based on reactivity (Gauggel et al., 1993; Wass and Belin, 1990). One common approach focuses on incubating the test chemical in the presence of a model nucleophile and then characterizing the reaction by measuring the depletion of the nucleophile, or the formation of adducts. A positive response for peptide reactivity is taken as an indication that a material may have the potential to act an allergen and cause sensitization. To facilitate the identification of pre- and pro-haptens, there has been interest in the development of reactivity assays that incorporate metabolic activation and/or autoxidative steps (Gerberick et al., 2009). A summary of the nucleophiles and analytical methods utilized in peptide reactivity assays are presented in Tables 1 and 2.

The nucleophiles selected for use in reactivity assays range from small molecules, such as butylamine, to full size native proteins (with the most commonly employed being HSA) (Alvarez-Sanchez et al., 2004b; Meschkat et al., 2001b; Roberts et al., 1983). There has also been interest in evaluating the reaction of test chemicals with proteins expressed directly in cultured cells (Hopkins et al., 2005; Natsch and Emter, 2008). In between these extremes, investigators have utilized a variety of peptides. Several groups have reported the use of 'designer peptides' that include single or several different reactive amino acids representing the range of soft to hard nucleophiles (Aleksic et al., 2009; Alvarez-Sanchez et al., 2004a; Bergstrom et al., 2006; Gerberick et al., 2004; Natsch and Gfeller, 2008; Nilsson et al., 2005). These designer peptides are not based on physiological proteins, or on known immunological macrostructures, but seek to optimize reactivity with the electrophile and enhance detection for a given analytical method. There have also been attempts by several researchers to use peptides based on physiologically relevant amino acid sequences. These have included the cellular peptide glutathione (GSH), which is the most abundant cellular thiol (Aptula et al., 2006; Bergstrom et al., 2007; Gerberick et al., 2004; Kato et al., 2003; Schultz et al., 2005). Glutathione plays a key role in conjugating and thus deactivating electrophilic toxicants (Deneke and Fanburg, 1989). The use of a peptide derived from the N-terminal chain of globulin, that contained all reactive amino acids except cysteine, was utilized by Alvarez-Sanchez et al. (2004a). Natsch et al. (2007) favored peptides with sequences derived from the substructures of physiologically reactive proteins. The sequences were selected based on proteomic analysis conducted to identify regions known to be reactive with electrophilic chemicals (Dennehy et al., 2006; Eggler et al., 2005; Wakabayashi et al., 2004). The selection of an appropriate peptide (nucleophile) for use in reactivity studies is an important consideration; a review of the potential advantages and disadvantages of several commonly reported nucleophiles is presented in Table 1.

The readout from reactivity assays can be qualitative, quantitative or both depending upon the method of analysis chosen. The selection of an appropriate analytical method is often determined

Table 1
Key protocol features of peptide reactivity assays – advantages and disadvantages of representative nucleophiles utilized. The nucleophiles selected for use in protein/peptide reactivity assays can range from short sequences, only a few amino acids long, up to full size native proteins. The researcher working in this area has a seemingly infinite array of nucleophiles to choose from when designing a protocol. The below are representative examples of commonly reported nucleophiles along with some key advantages and disadvantages associated with the use of each.

Key protocol choices	Advantages	Disadvantages	Representative reference
Peptides sequences:			
Single, predominate reactive nucleophile:			
Glutathione (GSH)	• Physiologically derived, biologically relevant peptide • Simplified system to target thiol reactivity, free N terminus • Well-described analytical methods available for kinetic analysis	• Applicability domain limited • Prone to dimer formation due to cysteine	Schultz et al. (2005)
AcRFAAXAA, (X = C, K, H) AcFAAXAA, (X = C, K, R, H, Y)	• Simplified system to target different applicability domains • Reaction conditions can be optimized per reactive nucleophile • Presence of chromophore can facilitate analyses (i.e., HPLC–UV)	• Prone to dimmer formation when cysteine present if DTT not utilized • Limited opportunity for nucleophile competition	Aleksic et al. (2009), Gerberick et al. (2004, 2007, 2009)
Multiple reactive nucleophiles:			
KRK and KR	• Simplified system of two reactive nucleophiles • Competition can be assessed in one reaction	• Bias toward lysine reactivity • Limited applicability domain, lacks other key nucleophiles	Reichardt et al. (2003)
PHCKRM	• Contains only the more reactive nucleophiles of interest • Competition can be assessed in one reaction	• Potential dimer formation due to cysteine • Multiple nucleophiles mutually affect reactivity • Increased analytical complexity	Bergstrom et al. (2006)
AcKYDCEQR, AcDSRCKDY, AcDSRCKDY, AcKRVCEEF, AcNKKCDLF, AcQANCYEE	• Sequences derived from physiologically reactive proteins • Nucleophile diversity (though limited) within each peptide • Potential for competition to be assessed in one reaction • Reported to have increased reactivity and solubility compared to similar peptides in literature	• Bias toward cysteine reactivity • Potential dimer formation due to cysteine • Multiple nucleophiles mutually affect reactivity • Three dimensional confirmations different from primary structure from which derived • Increased analytical complexity	Natsch et al. (2007)
VLSPADKTNWGHEYRMFQIG	• Globin based, physiologically derived sequence • All nucleophilic groups and N terminus present, except cysteine • Absence of cysteine prevents competing dimer formation • Competition can be assessed in one reaction	• Potentially relevant cysteine adducts not assessed • Multiple nucleophiles mutually affect reactivity • Three dimensional confirmations different from primary structure from which derived • Increased analytical complexity	Alvarez-Sanchez et al. (2004a,b)
Whole protein:			
Human serum albumin (HSA)	• Well characterized sequence • Biologically relevant, ubiquitous • Good solubility	• Increased analytical complexity • Not suitable for routine screening • Observed reactivity limited to conducive micro-environments under specific assay conditions	Alvarez-Sanchez et al. (2004a,b)

Abbreviations: DTT, d,l-dithiothreitol; HPLC–UV, high-performance liquid chromatography–ultraviolet light; peptide sequences, single letter amino acid abbreviations utilized.

by balancing quantitative/qualitative needs with resource considerations; a review of the potential advantages and disadvantages of several commonly reported methods utilized within reactivity studies is presented in Table 2. For the identification of potential skin sensitizers, there are two approaches that are commonly utilized and that have increasing large comparative datasets – high-performance liquid chromatography coupled with either UV detection (HPLC–UV), or mass spectroscopy (HPLC–MS). In the case of HPLC–UV, the loss/decrease of the peptide signal is monitored and the reaction is quantitatively expressed as the percent depletion of peptide when compared with the control (Gerberick et al., 2004). In this method of analysis, the loss of the peptide signal may be due to a variety of reaction mechanisms resulting in modification to the peptide. Covalent modifications are considered to be the most relevant reaction for skin sensitizing chemicals; however other competing reactions may take place such as oxidation and thiol dimerization. The relevance of these latter modifications for the acquisition of skin sensitization is unknown; it remains a possibility that they may play a role. If the confirmation of adduct formation is desired, HPLC–MS can be utilized. Detection via MS techniques can provide both a measurement of depletion and confirmation of adducts, although in the most basic form does not elucidate adduct structures (Aleksic et al., 2009; Gerberick et al., 2009; Natsch and Gfeller, 2008). With some additional effort the above analytical methods may be used to conduct experiments that can provide kinetic parameters to further quantify reactivity. An additional method, that is particularly suited to high-throughput kinetic analysis, is the detection of unreacted

Table 2
Key protocol features of peptide/protein reactivity assays – advantages and disadvantages of commonly reported analytical methodologies. The below are representative examples of commonly reported analytical methodologies utilized in peptide/protein reactivity assays. Advantages and disadvantages associated with the use of each are presented in the context of balancing quantitative/qualitative needs with resource considerations (assay through-put, cost, etc.).

Analytical detection methods	Advantages	Disadvantages	Representative reference
Spectrophotometric	• Easily adaptable for high-throughput dose–response and kinetics • Output is quantitative, measures depletion of peptide • Technically least demanding • Relatively inexpensive	• Use limited to specific nucleophiles with available fluorometric probe • Probe may compete with weak test chemical adducts complicating analysis • No confirmation of adducts, measurement is an aggregate of all reaction mechanisms	Schultz et al. (2005)
HPLC–UV	• Suitable for peptides containing a chromophore • Output can be qualitative and/or quantitative • Comparatively less technically demanding • Relatively inexpensive	• Peptide must contain a chromophore • Concentrations required can present solubility concerns • Co-elution of test chemical and peptide can complicate analysis • No confirmation of adducts, measurements are an aggregate of all reaction mechanisms	Gerberick et al. (2004)
LC–MS inclusive of a variety of detector arrangements	• Suitable for a wide variety of nucleophiles • Output can be qualitative and/or quantitative • Can confirm specific adduct formation • Lower reagent concentrations may be used to ease solubility concerns • Co-elution not a problem	• Comparatively more technically demanding • Relatively more expensive	Aleksic et al. (2009), Gerberick et al. (2009), Natsch et al. (2007)

Abbreviations: HPLC–UV, high-performance liquid chromatography–ultraviolet light; LC–MS, liquid chromatography–mass spectroscopy.

nucleophile targets by a probe followed by fluorometric or UV–Vis spectrometric detection (Schultz et al., 2005; Aleksic et al., 2009; Natsch and Gfeller, 2008).

From a hazard assessment perspective depletion measurements and/or the identification of adducts may provide sufficient information to identify chemical allergens and to rank them according to sensitizing potency. The elucidation of specific structures is valuable from a mechanistic point of view and may aid in the development of (Q)SAR models. Higher order MS techniques (tandem MS, MALDI-TOF-MS, nano electrospray tandem mass spectrometry [nano-ES-MS/MS]) and ^{1}H and ^{13}C NMR, often used in combination, have been applied to detect and characterize peptide and protein adducts with individual chemicals (Aleksic et al., 2007; Ahlfors et al., 2003; Alvarez-Sanchez et al., 2004b; Kato et al., 2003; Nilsson et al., 2005).

5. Skin and respiratory chemical allergens evaluated in reactivity assays

The majority of reports in the literature consider peptide reactivity assays within the context of identifying skin sensitizing chemicals (Aleksic et al., 2009; Gerberick et al., 2004, 2007; Kato et al. 2003; Natsch and Gfeller, 2008; Schultz et al., 2005). However, there are several studies that include assessment of both skin and respiratory sensitizers. A summary of studies demonstrating that protein reactivity is a common property of both types of allergen is provided in Table 3 and details of the independent investigations are provided below.

In work focused primarily on the development of peptide reactivity assays to identify skin sensitizers, several investigators have included respiratory allergens in their test chemical batteries and for some of these materials positive responses for peptide reactivity have been reported (Aleksic et al., 2009; Gerberick et al., 2004, 2007). These have included, among others, fluorescein isothiocyanate (FITC), glutaraldehyde, PA and TMA. In these cases, the authors focused on comparing peptide reactivity measurements with sensitization potential assessed in the LLNA. Consideration was not typically given to their potential to act as respiratory allergens. However, the data demonstrate that peptide reactivity (and activity in the LLNA) is a common feature for both skin and respiratory sensitizers.

Gauggel et al. (1993) considered the utility of measuring the binding of respiratory and skin sensitizers to HSA. Following test chemical incubation with albumin, a shift in retention time of the protein across an HPLC column was taken as a positive indication of adduct formation. Utilizing mass spectroscopy and electrophoretic techniques, the authors demonstrated that the shift could be attributed to conjugation with protein as opposed to denaturation or non-covalent interactions. The assay identified the skin allergens 2,4-dinitrochlorobenzene (DNCB) and formaldehyde as protein reactive. All respiratory allergens tested, with the exception of two aliphatic amines which may require metabolic activation, were also observed to covalently bind to HSA. These included anhydrides, chloramine-T, isocyanates, a platinum salt (ammonium tetrachloro-platinate) and the reactive dye Remazol Black. Non-sensitizers, representing a variety of chemical classes, were observed to lack reactivity to HSA.

The reaction of a di-peptide, containing both lysine and tyrosine, with a series of skin and respiratory allergens was investigated by Reichardt et al. (2003). The assay was based on detection of conjugates with the peptide via mass spectrometry after incubating the reaction mixture over several time points varying from 15 min to 4 days. Additionally, characterization of total peptide turnover with HPLC–UV following incubation with multiple concentrations of test chemical was conducted. This was expressed as a factor of reactivity (FR); defined as the amount of test chemical necessary to deplete 50% of the peptide. Adduct formation with the peptide was observed for both skin sensitizers (acetaldehyde, benzaldehyde, formaldehyde, glutaraldehyde, trinitrobenzene sulfonic acid and 2,4-dinitrofluorobenzene [DNFB]) and a limited number of respiratory sensitizers (PA and TDI).

The reactivity of a tri-peptide, containing one lysine and two tyrosines, with chemical allergens was considered by Wass and Belin (1990). The reaction was measured by HPLC–UV after incubation of the test material with the peptide. The percent depletion of peptide was converted to a peptide reactivity index based on a scale of 0–10 (0–100% peptide depletion). Respiratory allergens including anhydrides (HHPA, maleic anhydride, PA, TMA, tetrachlorophthalic anhydride) and isocyanates (HDI, isophorone

Table 3
Review of representative protein and peptide reactivity studies that consider both chemical skin and respiratory sensitizers. The majority of reports in the literature consider peptide reactivity assays within the context of identifying skin sensitizing chemicals. However, there are several studies that include assessment of both skin and respiratory sensitizers. The above summarizes those studies demonstrating that protein reactivity is a common property of both types of allergen.

Protein/ peptide	Characteristics	Analytical method	Measurement made	Chemicals tested	Key results	Reference
H-Lys-Tyr-OH	Dipeptide with one nucleophile more inherently reactive (lys) relative to the other (tyr)	HPLC–MS	Qualitative (+/−) Quantitative Factor of reactivity (FR) – amount of test chemical necessary to deplete 50% of the peptide	Respiratory sensitizers included PA and TDI	Adduct formation with the peptide was observed for both respiratory and skin sensitizers	Reichardt et al. (2003)
				Skin sensitizers included acetaldehyde, benzaldehyde, formaldehyde, glutaraldehyde, trinitrobenzene sulfonic acid and DNFB	The authors concluded that respiratory and skin sensitizers did not show any difference in reactivity to this dipeptide	
Lys-Tyr-Lys	Tri-peptide with two nucleophiles (Lys) more reactive than the other (Tyr)	HPLC–UV	Quantitative	Respiratory sensitizers included anhydrides, chloramine-T, isocyanates, platinum salt and reactive dyes	Respiratory sensitizers were observed to be reactive to the peptide	Wass and Belin (1990)
			Peptide reactivity index = % depletion of peptide divided by 10 to give a scale from 0 to 10	Skin sensitizers included DNCB, DNFB and formaldehyde	Failed to identify the skin sensitizers, reactive dyes and platinum salt (all known to be protein reactive based on recent studies)	
				Non-sensitizers – acids, bases and solvents	No reactivity was observed to the non-sensitizers	
AcRFAACAA, AcRFAAKAA, AcRFAAHAA, and GSH	Peptides containing 1 key nucleophile	HPLC-DAD	Quantitative, % depletion at fixed test chemical and peptide dose	>70 chemicals of known sensitization potential, as measured by the LLNA	Both respiratory and skin sensitizers showed potential to react with the peptides in this system	Gerberick et al. (2004, 2007)
				The majority of chemicals were skin sensitizers but the dataset included the respiratory sensitizers FITC, glutaraldeyhde, PA, and TMA		
Serum Albumin	Human	HPLC-DAD	Qualitative (+/−)	Respiratory sensitizers included anhydrides, chloramine-T, isocyanates, platinum salt and reactive dye	Respiratory and skin sensitizers were both observed to react to human serum albumin	Gaugell et al. (1993)
			Shift in retention time	Skin sensitizers – DNCB and formaldehyde	Non-sensitizers were not generally observed to be protein reactive	
				24 non-sensitizers		
Serum albumin and cell associated proteins	Co-incubation with bovine serum and U937 cells	Western blot	Quantitative densitometric analysis	Skin sensitizers – DNCB and DNFB	When co-incubated with serum and cell associated proteins, differential binding was observed	Hopkins et al. (2005)
				Respiratory sensitizers – FITC and TMA	Skin sensitizers bound preferentially to cellular proteins, while respiratory sensitizers bound to serum proteins All sensitizers bound to either protein when incubated alone with one or the other substrate	

Abbreviations: DNCB, 2,4-dinitrochlorobenzene; DNFB, 2,4-dinitrofluorobenzene; FITC, fluorescein isothiocyanate; HPLC–DAD, high-performance liquid chromatography–diode array detector; PA, phthalic anhydride; TDI, toluene diisocyanate; TMA, trimellitic anhydride.

diisocyanate, MDI, phenyl isocyanate, TDI, toluene-4-monoisocyanate) along with chloramine-T were observed to react with the peptide. In a few instances test materials considered by the authors to be respiratory sensitizers, such as certain reactive dyes, were not correctly identified as forming adducts. Furthermore, the skin sensitizers (DNCB, DNFB and formaldehyde) were observed to lack protein reactivity. DNCB, DNFB and formaldehyde are all known to covalently bind to lysine and tyrosine (Aleksic et al., 2009; Gerberick et al., 2004). The lack of reactivity observed by Wass and Belin (1990) may be the result of the test conditions,

specifically a pH of 7 with a reaction time of 10 min. In the more recent studies, an optimal pH of 10 was utilized for reaction with lysine and tyrosine and the incubation allowed to proceed for 24 h (Aleksic et al., 2009; Gerberick et al., 2004).

The studies above demonstrate only that protein reactivity is a common feature of both skin and respiratory allergens. However, it may be possible to distinguish between skin and respiratory allergens by observing preferences that emerge when reactivity with two or more different substrates are compared concurrently. In a report by Hopkins et al. (2005) *in situ* comparisons were made between the preference of different classes of chemical allergen for serum and cell associated protein binding. This was done by incubating chemical allergens in the presence of both substrates simultaneously. Experiments were conducted by culturing U937 cells (a human monocytic cell line) with bovine serum in the presence of respiratory (FITC or TMA) or skin (DNCB or DNFB) sensitizers for 1 h. The haptenated proteins were then resolved by Western blotting. The authors demonstrated that respiratory allergens bound preferentially to serum proteins, whereas skin sensitizers were found to bind selectively to cellular proteins. This differential binding was the result of true competition for peptide binding sites, as each allergen was found to bind covalently to cellular and serum proteins when incubated with each substrate alone. Additionally, the binding preferences emerged despite the fact that serum protein was present at concentrations almost 50-fold higher than was cellular protein (Hopkins et al., 2005). *In vitro* chemical reactivity assays are typically conducted with discrete, isolated peptides; under these conditions there is little opportunity for competitive binding between the nucleophile and potential chemical allergen. This is significantly different from *in vivo* exposures, where a chemical is presented with a variety of nucleophile targets within varying micro-environments. It remains possible, therefore, that observations of chemical reactivity under competitive binding conditions may prove useful for distinguishing between skin and respiratory chemical allergens.

6. Concluding comments

It is clear from both experimental studies and clinical experience that skin and respiratory chemical allergens have some discrete properties. Although they share several characteristics in common, they result ultimately in different qualities of immune response and different forms of allergic disease. When one considers the hurdles that a chemical allergen must negotiate in order to cause the acquisition of sensitization of either organ system, there are some commonalities, particularly with regard to fundamental requirements for induction of an immune response. The first of these is gaining access to relevant tissue compartments in which the initial immune recognition and triggering takes place (Basketter et al., 2007). The second of these is the requirement that chemicals interact with proteins/peptides in order to be recognized by the immune system (Lepoittevin et al., 1998). In this context, it is relevant to ask the following question: Can the nature of chemical interactions with proteins/peptides that occur at or near the site of exposure during these early stages of sensitization influence the quality of immune response that will evolve with time?

If one assumes that a chemical arrives in a relevant tissue compartment and in sufficient quantity, then interactions with peptides and proteins would seem to provide an opportunity for understanding, at a chemical level, the nature of different allergens. It is these interactions that form antigenic components, which then interact directly with the immune system to stimulate a biological response. It is clear that reactivity, either inherent or acquired, is a prerequisite for a chemical to act as an allergen. At first glance hapten-protein/peptide interactions would appear to be a purely chemical phenomenon. However, it has been demonstrated that the selective modification of different amino acid residues may explain the nature of biological responses to certain skin sensitizers (Alvarez-Sanchez et al., 2004a,b; Meschkat et al., 2001a,b). Similarly, some investigators have pointed to selective modification of specific amino acids or proteins as contributing to the ability of an allergen to result in either skin or respiratory sensitization (Ahlfors et al., 2005; Fleischel et al., 2009; Hopkins et al., 2005). While these observations are intriguing, further research is needed to determine if reactivity parameters – such amino acid specificity – can be applied broadly to distinguish between the form and quality of immune response that may develop.

Allergic diseases of the skin and respiratory tract remain important issues for both consumer product development and occupational/environmental health. There have been significant recent advances in the tools and methods available for the conduct of hazard characterization and risk assessment of skin sensitizers. In contrast, however, the development of methods to identify respiratory chemical allergens has proved more problematic. In both cases, there remains a reliance on animal models for hazard identification. There is considerable pressure globally from both governmental legislatures and non-governmental organizations to continue to eliminate wherever possible the need for experimental animals. Despite this there are currently available no widely recognized or validated *in vitro* methods for the identification and characterization of skin or respiratory chemical allergens.

Due to the significant level of cellular and molecular complexity associated with sensitization, it has become clear that no single *in vitro* test will likely be adequate in isolation for hazard characterization. For skin sensitization, a tiered testing approach has been proposed (Jowsey et al., 2006). There is now evidence to suggest that peptide reactivity would represent a central component of any such tiered strategy (Gerberick et al. (2008). The available peptide reactivity assays provide for the binary, qualitative identification of chemical allergens: a material either is or is not a sensitizer. A classification tree structure has also been proposed based on the results generated in the assay (Gerberick et al., 2007). The classification structure provides for some level of quantification of the chemical reactivity measured and relates this to potency classes of skin sensitizers. As with animal models of skin sensitization, the current iterations of these tiered approaches do not distinguish adequately between skin and respiratory allergens.

From risk assessment and risk management perspectives, it is necessary to separate chemicals based on their potential to act as skin or respiratory allergens. Investigations into the ability to distinguish between different classes of allergens based upon chemical reactivity may provide a fruitful area for research and exploitation. Ultimately, increased understanding of hapten-protein/peptide interactions will inform the development of tiered testing strategies for the identification and characterization of potential allergens.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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