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Contemporary Issues in Toxicology

Respiratory sensitization and allergy: Current research approaches and needs

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Abstract

There are currently no accepted regulatory models for assessing the potential of a substance to cause respiratory sensitization and allergy. In contrast, a number of models exist for the assessment of contact sensitization and allergic contact dermatitis (ACD). Research indicates that respiratory sensitizers may be identified through contact sensitization assays such as the local lymph node assay, although only a small subset of the compounds that yield positive results in these assays are actually respiratory sensitizers. Due to the increasing health concerns associated with occupational asthma and the impending directives on the regulation of respiratory sensitizers and allergens, an approach which can identify these compounds and distinguish them from contact sensitizers is required. This report discusses some of the important contrasts between respiratory allergy and ACD, and highlights several prominent *in vivo*, *in vitro* and *in silico* approaches that are being applied or could be further developed to identify compounds capable of causing respiratory allergy. Although a number of animal models have been used for researching respiratory sensitization and allergy, protocols and endpoints for these approaches are often inconsistent, costly and difficult to reproduce, thereby limiting meaningful comparisons of data between laboratories and development of a consensus approach. A number of emerging *in vitro* and *in silico* models show promise for use in the characterization of contact sensitization potential and should be further explored for their ability to identify and differentiate contact and respiratory sensitizers. Ultimately, the development of a consistent, accurate and cost-effective model will likely incorporate a number of these approaches and will require effective communication, collaboration and consensus among all stakeholders.

Keywords: Respiratory; Sensitization; Allergy; Animal models; Animal alternatives; Research approach

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An overview of respiratory sensitization and allergy

Allergy, in general, is defined as an adverse condition which is manifested following an immune response to an otherwise innocuous antigen. It is a member of a class of outcomes termed hypersensitivity reactions which are defined as harmful immune responses which result in tissue injury (Janeway et al., 1997). The resulting conditions that are of particular concern to industrial toxicologists include both respiratory allergy and allergic contact dermatitis (ACD). Respiratory allergy is a hypersensitivity reaction of the upper and lower respiratory tract to a protein or chemical xenobiotic. This hypersensitivity reaction is immediate, with clinical characteristics occurring within minutes to hours after xenobiotic exposure, and can include wheezing, breathlessness, tightness in the chest, bronchoconstriction, and/or nasal congestion. In extreme instances the reaction can elicit hypotension and life threatening anaphylaxis. In the general population, respiratory allergy is most frequently induced by environmental proteins including pollen, dust mite excreta and animal dander. However, in occupational settings, respiratory allergy can be mediated by industrial compounds including high molecular weight (HMW) compounds, such as protein detergents, and low molecular weight (LMW) chemicals. Due to their small size, LMW chemical allergens act as haptens which first react with proteins to create a complex that is then able to initiate an immune response.

Development of respiratory allergy to HMW and LMW compounds can contribute to the development of occupational asthma which is characterized by variable airflow limitation and/or non-specific bronchial hyperresponsiveness due to causes and conditions attributable to a specific work environment (Chan-Yeung and Malo, 1994; Karol, 1994). It is important to note that in addition to this immunological etiology, non-immunogenic agents such as irritants also play a significant role in the development of occupational asthma. In many cases, concurrent exposure to both allergens and irritants contributes to the condition. Clinical investigations have suggested that up to 20% of adult-onset asthma is caused by occupational factors and that 90% of these cases involve an immunological mechanism (Mapp, 2005). Furthermore, occupational asthma is the most prevalent occupational lung disease in developed countries. As a result, identification and characterization of compounds which have the potential to act as respiratory allergens are an important area of research for industrial toxicologists.

Not all compounds that provoke a specific immune response will have the potential to cause hypersensitivity of the respiratory tract. A larger number of compounds are associated with skin hypersensitivity and the development of ACD and are believed to have no sensitizing effect on the respiratory tract (Kimber and Dearman, 2005). Unlike respiratory allergy, ACD is an example of a delayed-type hypersensitivity reaction resulting from cell-mediated immune responses (Janeway et al., 1997). ACD is one of the most common occupational diseases with a number of compounds being implicated as causative agents, therefore, proactive identification and characterization of these compounds are also of considerable importance (Saary et al., 2005).

The development of hypersensitivity resulting in respiratory allergy and ACD consists of two distinct stages. The first is sensitization, which involves the development of an immune status, while the second is elicitation, which results in the clinical manifestation of allergy (Briatico-Vangosa et al., 1994). As a result, previously unexposed (naive) but susceptible individuals do not experience allergic symptoms the first time they are exposed to an allergenic protein or chemical. At a minimum it requires two exposures; however, in many cases it may require repeated exposures over weeks or months. During the initial encounters of a susceptible individual to an allergic compound, the compound is recognized as foreign by dendritic cells (antigen processing and presenting cells), presented to T cells, and a specific primary immune response is provoked which results in sensitization. This can be followed by the actual elicitation of allergy upon subsequent exposure of the sensitized individual to the same compound. Elicitation is mediated through the activation of an immune response and the resultant cellular signals which result in an inflammatory reaction and symptoms of the allergy. The nature and severity of the allergic reaction are dependent upon a number of factors including the genetic background of the individual, the characteristics of the allergen, as well as the route, duration and intensity of the exposure during both the sensitization and elicitation stages (Arts and Kuper, 2003; Arts et al., 2006).

Despite some general similarities, there are important mechanistic differences in the currently understood etiology of respiratory allergy and ACD (Fig. 1). Generally, respiratory allergy is classified as a type I hypersensitivity reaction involving IgE while ACD is a type IV hypersensitivity reaction which is mediated by T cells (Janeway et al., 1997). These hypersensitivities are thought to develop according to specific mechanisms that depend on the differential activation of functional subpopulations of T helper (Th) cells, namely, Th1 and Th2 cells. Development of respiratory sensitization and allergy has been associated with the preferential induction of a Th2 population of T lymphocytes. Th2 cells are characterized by the production of high amounts of interleukins (IL) -4, -10 and -13. The production of these cytokines favors humoral immune function and the stimulation and differentiation of B cells to produce IgE (reviewed in Dearman et al., 2003a). These antibodies bind to receptors on the surface of mast cells and basophils. Upon subsequent exposure to the allergen, these cells release various inflammatory mediators including histamine, leukotrienes and cytokines, which results in the immediate hypersensitivity of respiratory allergy. In addition to promoting IgE production, Th2 cytokines also promote the growth and differentiation of other cells involved in respiratory allergy including mast cells and eosinophils (reviewed in Kimber, 1996). Upon repeated exposure to allergenic compounds and the elicitation of respiratory allergy, extensive airway remodeling, mucus accumulation and chronic inflammatory responses may develop which contribute to the development of an asthmatic condition.

In contrast, the development of contact sensitization and ACD has been primarily associated with the induction of a Th1 population of T lymphocytes. These cells are characterized by

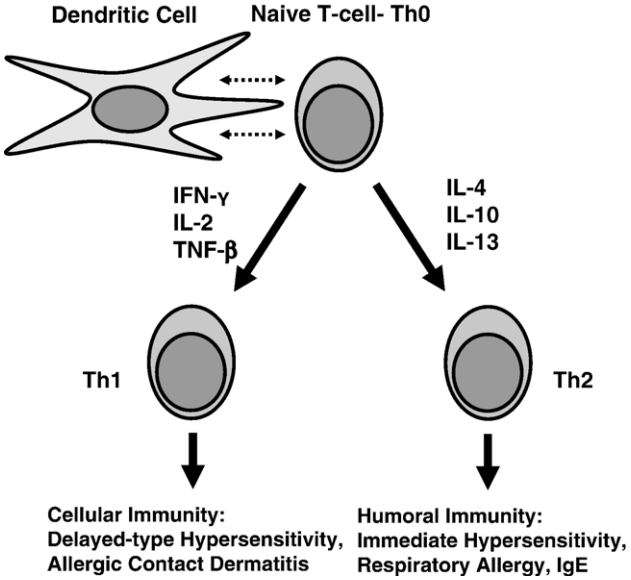

Fig. 1. Dichotomy of the T helper cell response in contact and respiratory sensitization. Dendritic cells internalize and process antigen and subsequently migrate to the draining lymph node where the antigen is presented to responsive T lymphocytes. This interaction can result in the proliferation and differentiation of T cells into a Th1 or Th2 cell lineage. Differentiation into Th1 cells is associated with the production of cytokines such as IL-2, IFN-γ and TNF-β and the development of cellular immunity associated with delayed-type hypersensitivity and allergic contact dermatitis. Differentiation into Th2 cells is driven by cytokines such as IL-4, IL-10 and IL-13 and is associated with the development of humoral immunity, immediate hypersensitivity and respiratory allergy. This dichotomy forms the basis for assays aimed at distinguishing contact and respiratory allergens.

the production of IL-2, interferon-gamma (IFN-γ) and tumor necrosis factor-β (TNF-β). Research has shown that the development of delayed contact hypersensitivity is specifically dependent on Th1 cells and the production of IFN-β (Diamantstein et al., 1988). The sensitization response is associated with the generation of memory T cells which are activated upon subsequent encounter with the antigen resulting in the hypersensitivity response. This reaction involves the activation of keratinocytes and the release of proinflammatory cytokines to recruit non-antigen specific T cells and monocytes to the site of contact which results in an acute inflammatory response (Roitt et al., 2001). Interestingly, IFN-γ produced by Th1 cells also antagonizes Th2 cell responses and the production of IgE, while IL-4 produced by Th2 cells antagonizes the development of Th1 cells. Furthermore, IFN-γ has been found to inhibit mast cell function in respiratory allergy, while IL-4 depresses the elicitation stage of ACD (reviewed in Kimber, 1996). Therefore, not only do the cytokines of each Th cell type promote the growth and differentiation of their lineage and the subsequent hypersensitivity response, they also antagonize the proliferation of the other cell population as a means of further directing the immune response.

The above distinction between respiratory allergy and ACD is of considerable importance from a hazard assessment and regulatory perspective. Researchers have explored a number of animal models and experimental approaches to identify compounds with the potential to cause respiratory allergy, however, none of the approaches are widely applied or fully accepted by the research community or regulatory agencies (Arts et al., 2006). In contrast, there are a number of guideline assays for the detection of compounds with the potential to cause contact sensitization and ACD. Given the general similarity of the sensitization response in respiratory allergy and ACD, it has been suggested that models for identifying the potential for contact sensitization could also be used for the assessment of the potential for respiratory allergy. However, due to the known mechanistic differences and the more serious health and regulatory implications for classification as a respiratory allergen, the accurate identification of these compounds and their distinction from compounds inducing ACD is critical. What is required is a consistent, systematic and accepted approach for assessing the respiratory allergy potential of both protein and chemical compounds. What follows is an overview of the current *in vivo*, *in vitro* and *in silico* approaches which have been used, proposed, or should be explored for the characterization of respiratory allergens.

Current approaches for the identification of respiratory allergens

In vivo approaches

The development of animal models to investigate the pathogenesis of respiratory allergy has paralleled our understanding of the human disease. The range of species used and the protocols of the experimental models vary greatly. Each model has its strengths and weaknesses, and many were developed to examine specific aspects of the human disease by exploiting unique characteristics of the experimental species. The strengths of animal models include the ability to use invasive experimental methods to explore pathogenic mechanisms, the accessibility of tissues and cells for complimentary *in vitro* studies, and the ability to control environmental and genetic factors that may influence disease development. Unfortunately, the fundamental limitation of all animal models studied to date is that none completely reproduce the complex physiological response observed in humans; rather, they reflect species-specific changes resulting from antigen provocation.

Many animal approaches have been developed with specific objectives, a single or limited number of xenobiotic molecules, and were not developed to meet broad hazard assessment goals. A limitation with this approach is that a full appreciation for the range of responses to unique or poorly characterized agents is not well understood, thereby restricting interpretation and definitive evaluation of these models for hazard assessment purposes. By example, investigators studying LMW chemicals have focused on understanding mechanisms using a limited battery of test chemicals or have focused on developing a more detailed assessment for a particular compound. Investigations into the allergenicity of proteins have also focused on a limited number of agents with the goal of understanding mechanisms and testing hypotheses for allergy, or asthma therapy and management. While it is realized that these approaches will aid in further defining mechanisms of action, a comprehensive assessment of the predictive value of the endpoints will not be realized until a wider panel of compounds has been examined.

In order to reproduce the key characteristics of human respiratory allergy, an animal model should include IgE-mediated responsiveness to allergen resulting in acute bronchoconstriction and increased airway resistance. Chronic inflammation of the airways with prominent eosinophilia, neutrophilia and elevated expression and secretion of Th2 cytokines should also be present along with persistent, reversible, airway obstruction, goblet-cell metaplasia/hyperplasia, mucous hypersecretion, and tissue remodeling including smooth-muscle hyperplasia and thickening of the collagen layer beneath the basement membrane. Clearly, no one endpoint or animal model can accurately predict or reproduce the entire range of human responses, therefore, it is critical to identify the key elements that best predict the allergic potential of protein and chemical xenobiotics. Although dogs, sheep, horses, and monkeys display a natural sensitivity to environmental allergens (Abraham, 2000) and represent viable models for researching respiratory allergy, the majority of the research for hazard identification and risk characterization has used the guinea pig, mouse or rat as research models, with each possessing advantages and disadvantages (Table 1). The following sections provide a generalized overview of the most prominent approaches in each of these species. For a more detailed description of these *in vivo* models the reader is directed to more comprehensive and focused reviews (Karol, 1994; Bice et al., 2000; Pauluhn and Mohr, 2005; Arts and Kuper, 2007; Ward and Selgrade, 2007b).

Guinea pig models

The guinea pig has been used as an animal model for respiratory allergy research for nearly a century (Karol, 1994). Guinea pigs possess a number of characteristics which make them a suitable species for predicting the respiratory allergy potential of xenobiotics. The main advantage is their ability to display respiratory reactions similar to those observed in human allergic diseases (Briatico-Vangosa et al., 1994). Unlike other rodent models, the lung is the target organ of the hypersensitivity response in guinea pigs (reviewed in Karol, 1994). These animals possess a well-developed bronchial smooth muscle compartment which can respond vigorously to antigen and facilitates the detection of airway constriction in both early and late pulmonary hypersensitivity responses (reviewed in Pauluhn and Mohr, 2005). Furthermore, the pulmonary inflammatory response is comprised of both eosinophilic and neutrophilic infiltrates, similar to that observed in the human condition. However, there are also a number of limitations with using guinea pigs for respiratory allergy research, including the small number of characterized inbred strains and the limited number of species-specific reagents which restricts the investigation into genetic influences and the cellular and molecular mechanisms of action. In addition, the major class of antibody responsible for the anaphylactic response in guinea pigs is IgG1, whereas in humans IgE is the predominant class (Pauluhn and Mohr, 2005). Furthermore, guinea pigs are sensitive to developing respiratory tract infections and have a higher propensity to exhibit airway hyperreactivity which can complicate data interpretation and inhibit inter-laboratory reproducibility.

Due to the advantages and disadvantages listed above, the majority of the research with guinea pigs has focused on methods which seek to identify respiratory allergens based on the elicitation of pulmonary responses in sensitized animals. Extensive research has been conducted on assessing the pulmonary allergic potential of both LMW chemicals and proteins. These investigations have used a wide variety of sensitization approaches including inhalation, intratracheal instillations, and intraperitoneal injections. Challenge exposures have involved both inhalation and intratracheal instillations to proteins and chemicals. Subsequent assessment of respiratory allergy has primarily been performed by monitoring pulmonary reactions and allergic antibody (IgG) production. One notable approach is the guinea pig intratracheal test (GPIT) in which animals are administered the test material and the resultant IgG1 antibody

Table 1
Advantages and disadvantages of guinea pig, mouse and rat models for respiratory allergy research

Species	Advantages	Disadvantages
Guinea pig	• Exhibit respiratory reactions similar to humans • Lung is the target organ of hypersensitivity reaction • Possess well developed bronchial smooth muscle • Eosinophilic and neutrophilic lung infiltrates similar to humans	• Few characterized in bred strains • Limited number of species specific reagents • IgG1 is the effector antibody • High incidence of non-antigen mediated pulmonary hyper reactivity
Mouse	• Well-characterized immune system • Wide availability of species-specific reagents • IgE is the effector antibody • Exhibit eosinophilic lung infiltration • Fully sequenced genome, genomic tools • Characterized inbred strains and genetic models • Cell lines available for *in vitro* research	• Poorly developed bronchial musculature • Vasculature is the target of the hypersensitivity response • Small size can create experimental difficulty in measuring endpoints
Rat	• Extensive use in toxicology research • IgE is the effector antibody • Exhibit eosinophilic lung infiltration • Larger size is more amenable to measurement of pulmonary reactions and cellular responses • Genomic information available	• Weak bronchoconstrictor compared to guinea pig • Limited number of species-specific reagents • Less annotated genome compared to mouse • Fewer in bred strains and genetic models available when compared to the mouse

response is measured as an index of sensitization. This approach has been used to determine the relative potencies of detergent enzymes and was subsequently used to set occupational exposure guidelines (Ritz et al., 1993; Robinson et al., 1998). However, a clear and consistent association between IgG titers and respiratory reactions has not been established which has limited the use of this endpoint in as a predictor of respiratory allergy potential (Bice et al., 2000; Pauluhn and Mohr, 2005). Alterations in breathing parameters such as respiratory rate, minute volume, tidal volume, peak expiratory flow, inspiratory and expiratory times and a flow-derived estimation of airflow restriction (enhanced pause, Penh) are often used for quantitative assessment of allergen induced airway hyperreactivity (Thorne and Karol, 1988; Pauluhn, 1994, 1997; Pauluhn and Mohr, 1994). Both restrained and unrestrained plethysmographic systems have been used to measure these respiratory parameters in response to a range of respiratory allergens including detergent enzymes, acid anhydrides and isocyanates (Gerlach et al., 1989; Kimber et al., 1996; Pauluhn, 1997; Pauluhn et al., 2002a,b). It must be recognized that these are indirect indicators of altered pulmonary function and in some cases can occur in the absence of bronchoconstriction and measurable airflow restriction. For example, the use of Penh as an accurate estimator of airway resistance has been met with controversy due to inconsistent correlations between these endpoints (Bates et al., 2004). This uncertainty can be eliminated through supplemental testing using more invasive techniques which make use of anesthetized animals and equipment to perform forced ventilatory maneuvers.

Although guinea pigs have served as a valuable research model for hazard identification and risk characterization of respiratory allergens, the cost and time associated with these investigations can be prohibitive. Furthermore, the lack of immunobiological reagents specific for this species has limited the ability to conduct more mechanistic research (Robinson et al., 1998). This has driven the development of predictive models using mice and rats which are more amenable to mechanistic investigations of both the sensitization and elicitation stages of respiratory allergy (Kimber et al., 1996; Robinson et al., 1998).

Mouse models

While guinea pig models have focused extensively on pulmonary reactions during the elicitation stage of respiratory allergy, mouse models have explored more mechanistic characterization of both the sensitization and elicitation stages. Investigations into the cellular and molecular mechanisms of action in mice have been facilitated by their well-characterized immune system, the availability of species-specific reagents, a fully sequenced genome, and various genetically modified models. Mice also exhibit a number of similarities to humans in their response to respiratory allergen including the development of IgE antibody, eosinophilic lung inflammation, mucus hypersecretion and airway hyperresponsiveness (reviewed in Epstein, 2006). However, mice also possess many limitations as their vasculature is the actual target of the anaphylactic response, they possess a poorly developed bronchial musculature, and their small size can make it difficult to detect pulmonary reactions associated with the elicitation of respiratory allergy or to achieve the appropriate number of cells required for analyses (reviewed in Karol, 1994). However, with improving technologies it has been possible to monitor cellular and molecular responses with a limited number of cells and to more accurately measure respiratory responses in mice using both plethysmography and forced ventilatory maneuvers.

Although pulmonary endpoints are important for characterizing the response to respiratory allergens, the technological requirements, study duration and ultimate cost of performing these assays can be prohibitive. Therefore there have been extensive efforts put forward to assess the sensitized state of animals to more efficiently characterize respiratory allergens. Due to the general similarity in the acquisition of a sensitized state for both contact and respiratory sensitizers, it has been proposed that well-established contact sensitization assays, such as the mouse local lymph node assay (LLNA), be used as a first tier for characterization of LMW chemicals (Dearman and Kimber, 1999; Arts and Kuper, 2007). The LLNA is a mechanistically based assay that monitors the induced proliferative response of lymphocytes in the draining lymph nodes during sensitization (OECD, 2002). This proliferative response is common to both contact and respiratory sensitizers, although the resultant T cell populations differ, with contact sensitizers inducing a Th1 response and respiratory sensitizers inducing a Th2 response. It is recognized that only a small number of compounds which test positive in this assay are actual respiratory sensitizers, therefore, additional discriminating approaches are required to further characterize the respiratory allergy potential. Such approaches have included the mouse IgE test and cytokine profiling. These approaches take advantage of what is currently understood about the mechanisms of action for contact and respiratory sensitizers with respect to the dichotomy of the T helper cell response (i.e., Th1 versus Th2).

The mouse IgE test is a quantitative assay that categorizes a compound as a respiratory allergen based upon its ability to induce serum IgE, the class of antibody that is known to mediate immediate hypersensitivity reactions in both mice and humans. This assay has been used within the context of LMW chemicals; however, the mouse intranasal test (MINT) is founded on a similar approach which relies on measuring the antibody response to intranasally administered proteins (Robinson et al., 1998). Although many investigators have reported increases in serum IgE in response to known respiratory sensitizers, there are exceptions and inconsistencies with the approach that have limited acceptance of this endpoint. By example, the known contact sensitizer dinitrochlorobenzene (DNCB) has been shown to induce minimal, yet significant, increases in IgE production, thereby constituting a potential false positive (Farraj et al., 2004; Selgrade et al., 2006). In addition, symptomatic individuals have not demonstrated a consistent requirement for IgE in mediating respiratory allergy responses to certain diisocyanates suggesting that IgE may not be the sole contributor to the condition (reviewed in Holsapple et al., 2006; Arts and Kuper, 2007). Research has also revealed poor correlations between the degree of IgE induction and alterations in

pulmonary function (Vanoirbeek et al., 2003). Similar results have been noted for the assessment of protein allergenicity with allergic and 'non-allergic' proteins yielding IgE responses inconsistent with human experience (reviewed in Thomas et al., 2005b). It is important to note that these inconsistencies may be due to numerous factors including technical difficulties in assessing IgE levels (total versus antigen-specific IgE), non-optimized dosing regimens, material purity and source, vehicle effects, species-specific responses, or a lack of understanding of the complete mechanisms involved in mediating respiratory allergy. For example, although IgE is a dominant mediator of the hypersensitivity response, there is research which indicates that IgG1 may also play a contributing role in mice (Miyajima et al., 1997), suggesting the need for multiple endpoints in the assessment of respiratory allergy potential.

Considering the general mechanism for IgE production many investigators have also conducted targeted screens for specific cytokines, an approach referred to as cytokine profiling (Dearman et al., 2003a). This approach attempts to discriminate contact and respiratory sensitizers on the basis of the cytokine profiles of the Th1 and Th2 cellular responses. The most common profile of cytokines associated with a Th2-mediated immune response includes some combination of IL-4, -5, -10, -13, while Th1 responses have been associated with induction of IFN-γ, TNF and/or IL-2. Research has indicated that known respiratory allergens elicit a more robust, but not completely distinctive, profile consistent with Th2-cell activation (Vandebriel et al., 2000; Van Och et al., 2002; Dearman et al., 2003a,b). However, as with the IgE test, a number of inconsistencies have been noted when examining a broader range of compounds (Ulrich et al., 2001; Selgrade et al., 2006). As a result, these approaches have yet to receive broad acceptance, however, additional standardization of both cytokine profiling and the IgE test may increase their predictive value and promote their use in a tiered- or weight of evidence-based assessment approach.

In addition to research involved in characterizing the sensitization stage, a number of sensitization-challenge protocols have recently been established using mice (Farraj et al., 2003, 2006; Sailstad et al., 2003; Duez et al., 2004; Vanoirbeek et al., 2006, 2004; Pauluhn and Mohr, 2005; Selgrade et al., 2006; Arts and Kuper, 2007). An important consideration for these experiments (as well as for guinea pig and rat studies) has been the route of exposure selected for sensitization which has included inhalation, intratracheal or intranasal instillation, dermal or intraperitoneal routes (Arts and Kuper, 2007). Each of these approaches has advantages and disadvantages in terms of relevance and efficacy which should be taken into account when considering the purpose of the study (Pauluhn et al., 2002a; Arts and Kuper, 2003). Furthermore, the route of exposure may influence the subsequent T helper cell response (Iwasaki and Kelsall, 1999). Regardless of the sensitization regimen, sensitization-challenge assays typically monitor a host of respiratory parameters in response to an inhalation, intranasal or intratracheal challenge including, but not limited to, respiratory rate, inspiratory and expiratory flow rates, and Penh (Vanoirbeek et al., 2004, 2006; Pauluhn and Mohr, 2005; Selgrade et al., 2006; Arts and Kuper, 2007). These endpoints can be monitored specifically, in response to the putative allergen, or non-specifically, to an agent such as methacholine. The non-specific challenge with methacholine provides an assessment of airway hyperreactivity that circumvents the potential confounding irritant effects of the test material, an effect which is of particular concern for LMW chemicals. Lung function measurements are typically complemented with histopathological analysis of the lung and nasal tissue combined with examination of biochemical and cellular parameters of the bronchoalveolar lavage fluid (BALF). Alterations in these parameters are often associated with sustained pulmonary inflammation which can be characterized by changes in tissue cytometry and BALF including endpoints such as lactate dehydrogenase, protein, eosinophil peroxidase and myeloperoxidase (Pauluhn et al., 2002a). In some instances the histopathological and BAL analyses have been conducted in the absence of pulmonary function tests as these approaches do not require specialized equipment (Farraj et al., 2003, 2006; Sailstad et al., 2003; Duez et al., 2004). However, the direct relationship of these endpoints to respiratory allergy needs to be interpreted with appropriate scientific judgment. Although extensive research and progress has been made on developing these challenge protocols, they have not been extended beyond a small set of known respiratory allergens and have not been used to determine relative potency and examine thresholds. Furthermore, a comprehensive inter-laboratory comparison of the results obtained with the diverse array of sensitization and challenge protocols has yet to be conducted.

One important consideration that cannot be overlooked when developing mouse models of respiratory allergy is strain selection. Comparative studies have indicated that not all mouse strains respond similarly to sensitization and/or challenge with a respiratory allergen. Strain differences have been noted on various endpoints including the type and degree of cellular immune response, the production of IgE, and the degree of bronchial hyperreactivity (Brewer et al., 1999; Seitzer et al., 2005). Although strain differences are likely not to be unique to the mouse, the availability of a wide range of inbred strains accentuates the issue. Some of the more commonly used strains have included the high-IgE-responding BALB/c, A/J and BDF1 mice, however, responses across these and other strains can differ dramatically and remain a significant hurdle for experimental reproducibility and the development of a consensus approach.

Rat models

In recent years, there has been an increase in the use of the rats for respiratory allergy research. Much of this is due to the extensive use of rat strains in general toxicology studies; however, the rat also possesses additional advantages when compared to other animals models (reviewed in Karol, 1994; Pauluhn and Mohr, 2005). These include a number of similarities to the human condition including the production of IgE in response to allergens, the accumulation of neutrophils, lymphocytes and eosinophils in the lung lavage and tissue, and the characteristic elevation of Th2 cytokines and a reduction in Th1 cytokines. Furthermore, the larger size of the rat, when

compared to that of the mouse, allows for greater ease in the measurement of breathing parameters and in obtaining sufficient quantities of tissues or fluids for molecular and cellular analyses (Pauluhn and Mohr, 2005; Martin and Tamaoka, 2006). In terms of disadvantages, although the rat displays an airway hyperactivity response to agents such as methacholine, when compared to the guinea pig the rat is a weak bronchoconstrictor (Bice et al., 2000). From a molecular standpoint, genome sequencing efforts and functional gene annotation are more limited for the rat when compared to the mouse; although they are more complete when compared to the guinea pig. In addition, the number of immunological reagents and genetic models available to mechanistically characterize allergic responses in the rat is limited when compared to that of the mouse.

Respiratory allergy research in rats has involved assays examining both sensitization and elicitation responses and has largely utilized the high-IgE-responding Brown Norway strain in conjunction with approaches described for guinea pigs and mice. Investigators have demonstrated that the LLNA can also be applied for the detection of sensitizers in rats (Arts et al., 1996; Vento et al., 1996). These results have been followed by studies which have indicated that Brown Norway rats are also suitable for use in the IgE test after dermal exposure to the well-characterized respiratory allergen, trimellitic anhydride (TMA) (Arts et al., 1997). The results of experiments with TMA have been corroborated by a number of other researchers with varying sensitization protocols and routes of administration (Warbrick et al., 2002a,b; Zhang et al., 2002). Therefore, a combination of these two assays offers an approach to characterize the respiratory sensitization potential of LMW chemicals as in the mouse, although the limitations observed for these endpoints in the mouse also extend to the rat. To further complement this approach, subsequent studies have examined the elicitation stage of respiratory allergy in response to TMA after both dermal and inhalation sensitization followed by inhalation challenge and subsequent examination of lung histopathology and analysis of airway hyperresponsiveness to the sensitizing compound or a non-antigenic stimulus such as methacholine (Arts et al., 2001, 2003). These studies have revealed that airway parameters in rats can be used to further characterize the respiratory allergy potential of a compound; however, as with the mouse, the sensitivity and dynamic range for many of the responses are limited and the ability to distinguish allergic responses from irritant effects can be difficult. A promising aspect of this research was the identification of a no-observed effect level (NOEL) for TMA, a value that is highly desired for effective risk assessment of respiratory allergens (Arts et al., 2004).

Similar approaches using the Brown Norway rat have been applied to research on the respiratory allergy potential of diphenylmethane diisocyanate (MDI) after both dermal and inhalation sensitization followed by inhalation challenge. These studies used repeated inhalation challenge to more realistically mimic the human exposure situation and to increase the sensitivity and robustness of the model (Pauluhn, 2005; Pauluhn et al., 2005). Results indicated that serum IgE and respiratory parameters may not be the most reliable endpoints for evaluating respiratory allergens, and analyses of conventional histopathology and BALF should also be conducted.

A number of varied approaches with rats have also been applied to research on the respiratory allergy potential of proteins including ovalbumin, house dust mite and pollens (Ward and Selgrade, 2007a). Research on proteins has focused on characterization of physiological responses to respiratory allergens and interactive effects of environmental contaminants with less emphasis on hazard identification. As with the mice and guinea pig models, rat models for both proteins and chemicals suffer from inconsistencies across laboratories which are likely due to differences in doses, dosing regimens, adjuvants, sampling times and methodologies. Furthermore, definitive endpoints that are predictive and consistent across compounds and laboratories have yet to be fully characterized and agreed upon. Although research with rat models has provided valuable data on the characterization of various respiratory allergy endpoints in response to a small number of well-characterized respiratory allergens, the true value of these approaches for the prediction of respiratory sensitization potential will be realized upon evaluation of a more diverse array of compounds.

In vitro approaches

Although animal models have offered significant insight into the identification of both contact and respiratory sensitizers and allergens, a continued challenge has been the development of *in vitro* approaches to reduce, refine and replace the use of animals. Investigations into these approaches have been facilitated by significant advances in the understanding of the cellular and molecular mechanism through which allergic sensitization to chemicals is induced and regulated. This has allowed investigators to explore the use of *in vitro* approaches such as novel cell culture systems and chemical reactivity assays. It is important to note that most of the research to date has focused on the development of *in vitro* models for the detection of contact sensitizers; however, these approaches are also likely to be applicable for the detection of respiratory sensitizers. Subsequent to the establishment and acceptance of *in vitro* models for detecting sensitization, additional research will need to be applied to assess their ability to discriminate between contact and respiratory sensitizers (Toebak et al., 2006). Alternatively, *in vitro* models may serve a role in a tiered assessment approach for sensitization that will involve further characterization of the response through *in vivo* studies to differentiate respiratory from contact sensitizers.

Many recent studies have focused on the potential use of cultured dendritic cells as an alternative to *in vivo* studies. Dendritic cells are a distinct group of leukocytes characterized by their unique morphology and ability to initiate immune responses by processing and presenting antigens to T cells (Ryan et al., 2005). During this process these cells undergo a maturation process in which they acquire a phenotype of professional antigen presenting cells which is accompanied by alterations in cell surface markers, the expression of various cytokines and activation of T cells. Researchers are using the

characteristics of this maturation process as potential molecular endpoints for the identification of sensitizing compounds.

Although both the skin and lung airway mucosa possess resident dendritic cells, the majority of studies conducted to date have utilized human peripheral blood mononuclear cell-derived dendritic cells (PBMC-DC) due to their relative ease of extraction and the ability to obtain larger quantities of cells (Casati et al., 2005). In addition to having typical DC morphology, PBMC-DC express surface markers consistent with bone-marrow-derived DC and are capable of inducing proliferative responses in resting T cells (Romani et al., 1996). A number of researchers have monitored the response of PBMC-DC to various sensitizers and reported alterations in endpoints such as receptor-mediated endocytosis (Becker et al., 1997), surface marker expression (Aiba et al., 1997; Hulette et al., 2002), and gene expression responses (Pichowski et al., 2000; Kimber et al., 2001; Ryan et al., 2004; Hansen et al., 2005) that were not mediated by weak or non-sensitizing compounds. These data support the use of these cells and endpoints as a potential *in vitro* approach for assessing the sensitizing potential of compounds. However, there are limitations to this model including donor to donor variability as well as the variations in cell isolation and culture techniques which may limit the reproducibility of results within and between laboratories (Casati et al., 2005). These constraints will need to be addressed if the use of PBMC-DC is expected to progress as a potential *in vitro* model.

In order to circumvent the limitation of donor variability for PBMC-DC, researchers have explored the use of dendritic-like cell lines. These cell lines offer the advantage of providing a continuous and more consistent population of cells which should increase experimental reproducibility and allow for more mechanistic studies through cellular manipulations. A number of mouse and human dendritic-like cell lines have been investigated including the murine XS52 DC cell line and the human myeloid cell lines KG-1, THP-1 and MUTZ-3 (Ryan et al., 2005; Larsson et al., 2006). XS52 cells have been shown to increase MHC class II expression in response to sensitizers but not irritants (Herouet et al., 2000), while human THP-1 cells displayed enhanced expression of CD86 and internalization of MHC class II molecules upon exposure to allergens (Ashikaga et al., 2002; Yoshida et al., 2003). These data suggest that the use of these cell lines may represent a viable approach for the identification and characterization of sensitizers. However, one persisting limitation for both PDMC-DC and continuous dendritic-like cell lines is the lack of sensitivity and dynamic range in response to potent allergens for the endpoints examined to date (Casati et al., 2005). To address this, a number of researchers are applying microarray-based approaches in an effort to identify more dynamic and predictive gene expression endpoints (Ryan et al., 2004; Schoeters et al., 2006).

Additional non-cell-based *in vitro* approaches are also being explored as alternatives to animal models including the assessment of chemical–protein reactivity. Many chemical sensitizers act as haptens which must react with host proteins as a prerequisite to their ability to function as allergens. These chemicals often possess electrophilic properties enabling them to react with nucleophilic amino acids such as lysine, cysteine and histidine to form covalent bonds. The resultant hapten–protein conjugate is then able to elicit an immune response. A number of researchers have explored the use of peptide reactivity assays to aid in the identification of respiratory allergens by identifying chemical–protein conjugates (Gauggel et al., 1993; Kato et al., 2003; Gerberick et al., 2004). One important consideration of this approach is that many chemicals exist as prohaptens which must first be metabolized prior to acquiring the ability to act as sensitizers. Therefore, assays aimed at using peptide reactivity should consider incorporation of a metabolic activation step in the assessment approach. Overall, the development and use of peptide reactivity assays may be a valuable component in a strategy that requires rapid screening and candidate prioritization; however, such an approach may be less relevant to research efforts aimed at compound characterization.

In summary, a number of *in vitro* approaches for assessing the sensitization potential of compounds are currently under investigation. This research has been facilitated by significant advances in our understanding of the cellular and molecular mechanisms through which allergic sensitization to chemicals is induced and regulated. Due to the complex nature of the immune system, many *in vitro* assays will only be able to assess one aspect of the multi-step process required to achieve sensitization. Therefore, the successful implementation of an *in vitro* approach may best be achieved through the application of an array of assays and integration of the results as proposed by Jowsey et al. (2006). As of yet, no attempts have been made to assess the ability of these *in vitro* models to distinguish between contact and respiratory sensitizers, however, this will be a key factor in determining the ultimate utility of these approaches in the assessment of respiratory allergy potential. It is important to realize that these *in vitro* approaches are still in the early stages of development and their successful implementation into an accepted test strategy will require continued research, anchoring to *in vivo* endpoints, extensive standardization, and collaboration between researchers.

In silico approaches

The search for alternatives to animal models for the identification of respiratory allergens has also explored the use of *in silico* approaches. These approaches identify respiratory allergens using structure–activity relationships (SAR), which involve a combination of computational chemistry/biology and statistics to identify a link between structure and biological activity (Graham et al., 1997; Fedorowicz et al., 2005). SAR models rely on the use of databases containing physical–chemical properties of experimentally examined compounds and evaluate their relationship to bioactivity. This information is then used to evaluate and predict the bioactivity of previously untested compounds (Fedorowicz et al., 2005). In the case of chemicals, SAR models are primarily based on chemical family structural alerts and reactivity of functional groups with proteins. SAR modeling software applications, including DEREK, TOPKAT, MULTICASE and TIMES-SS have been developed and are used in many toxicological applications (Fedorowicz et al., 2005; Patlewicz et al., 2007). Predictions of protein

respiratory allergy potential have involved the use of bioinformatic approaches to assess the amino acid sequence or structural motif similarity to known protein allergens and are available through a number of allergen databases (reviewed in Thomas et al., 2005a). Although initial evaluations of these *in silico* predictive models have been promising, currently there is insufficient confidence to definitively indicate the absence of sensitizing or allergic potential (Fedorowicz et al., 2005; Jowsey et al., 2006). As a result, these models will most appropriately be used in prioritization of lead candidates, in tiered testing approaches, or to address issues in the absence of any specific data (Sarlo and Clark, 1992; Fedorowicz et al., 2005; Jowsey et al., 2006).

As the predictive modeling software is continually refined and incrementally improved, the size, scope and complexity of the available databases will ultimately be the factor limiting *in silico* evaluation of new chemicals and proteins. Although public databases of allergic proteins and chemicals exist for use in these evaluations, the number of chemicals and proteins known to cause respiratory allergy is small in number, severely limiting the utility of these approaches for prediction of respiratory allergenicity (Fedorowicz et al., 2005; Thomas et al., 2005a; Holsapple et al., 2006). Many of the current approaches have used information on contact sensitizers in the development of their predictive models which may severely limit the accurate classification and distinction between contact and respiratory sensitizers (Graham et al., 1997). Despite this limitation, *in silico* models should continue to be explored as alternatives to animal models and will certainly benefit from the development of more robust and predictive *in vivo* and *in vitro* approaches for identifying respiratory allergens. These approaches will increase the number and diversity of compounds on which the *in silico* models will be developed and will further enhance our understanding of the underlying mechanisms which can be incorporated to further refine the predictive models.

The need for a model/approach to identify respiratory allergens

Despite extensive research with a variety of animal models and our current understanding of the molecular, cellular and physiological events associated with respiratory sensitization and elicitation, there are currently no widely applied or fully accepted approaches to identify compounds with the potential to cause respiratory allergy (Arts et al., 2006). Examination of published approaches reveals considerable differences with regards to animal species utilized, route of exposure, method of administration, protocols for induction and elicitation of allergic responses, endpoints monitored, and criteria for indicating a positive response (Karol, 1994). One factor limiting the ability to compare results across laboratories is the diverse array of sensitization and challenge protocols employed by different researchers, even when using the same *in vivo* animal model. Additional gaps involve the absence of data on thresholds for sensitization and elicitation, and the lack of negative control compounds in these assays. Although the current dogma suggests that the thresholds for elicitation are lower than that for sensitization, there is uncertainty surrounding this view (Holsapple et al., 2006), and at present, there has been no comprehensive examination of the dose–response relationship between these stages of respiratory allergy. Furthermore, assays aimed at addressing HMW and LMW compounds have largely been developed independently of one another, however, each may benefit from a more harmonized approach.

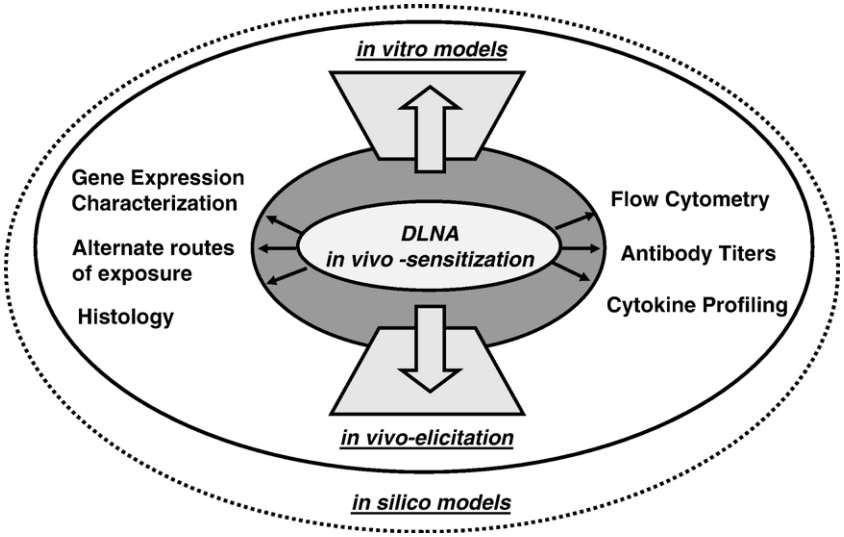

Fig. 2. Potential integrated approach for respiratory allergy research. Based on the wide acceptance and utility of the LLNA, a draining lymph node assay (DLNA) approach for assessing *in vivo* sensitization potential may be an important starting point for the characterization of putative respiratory allergens. Such an approach may be enhanced through exploration of alternate routes of exposure and application of additional endpoints including gene expression responses, cell surface markers, histology, and antibody responses. Mechanistic insights and putative biomarkers can subsequently be leveraged in the development of more predictive *in vitro* assays. In addition, these endpoints and approaches can be extended towards the development of more consensus and reproducible *in vivo* elicitation assays. Each approach will aid in the development of improved *in silico* approaches and ultimately lead to an integrated tiered approach for the characterization of potential respiratory allergens.

From a regulatory perspective there is a pressing need to develop a model for the definitive identification of respiratory allergens. Current European Union (EU) labeling for respiratory sensitizers involves the symbol Xn, as an indication of harm, and the risk phrase R42 indicating the potential to cause sensitization by inhalation. These classifications are applied in the absence of any validated animal assays to truly assess the contribution to respiratory allergy. The need for a model is further highlighted by the recently implemented EU regulatory framework for the registration, evaluation and authorization of chemicals (REACH). Under this legislation, respiratory sensitizers will be included among chemicals of high concern, including carcinogens, mutagens and reproductive toxicants, and at one point were being considered for authorization under REACH which involves more restricted use and chemical substitutions where appropriate (European Commission, 2006a,b). In addition, under the EU authorization directive 91/414/EEC concerns over the contact sensitization potential of pesticide active substances have been linked to the potential for respiratory sensitization without a distinction between the two conditions (European Commission, 2006a,b). Within the Globally Harmonized System for the Classification and Labeling of Chemicals (GHS), classification as a respiratory sensitizer relies heavily on human evidence and proposed animal studies include the mouse IgE test and pulmonary responses in guinea pigs despite the lack of consensus and guidance for these assays (United Nations Economic Commission for Europe, 2007). The drafting and impending implementation of these directives further highlights the need to develop assays which are able to identify compounds that are respiratory allergens and differentiate them from contact sensitizers.

In contrast to respiratory allergy, there are a number of guideline assays for the detection of contact allergens including the guinea pig maximization test (GPMT) and Buehler assay (GPBA) (OECD, 1992), as well as the mouse LLNA (OECD, 2002). In addition to identifying contact sensitizers, it has been proposed that the LLNA is also able to identify respiratory sensitizers, despite the fact that these compounds are not associated with contact sensitization (Vanoirbeek et al., 2003; Holsapple et al., 2006). However, the LLNA is unable to distinguish contact and respiratory sensitizers, it is not applicable to proteins, and research has not been conducted to examine if compounds which are negative in the LLNA could still have the potential to act as respiratory sensitizers. Despite these hurdles, as an accepted and widely used assay for contact sensitization with several advantages, the mouse LLNA may represent a valuable starting point for the development of assays to characterize the potential for compounds to elicit respiratory sensitization (Dearman and Kimber, 1999; Arts and Kuper, 2007). However, it is important to realize that a positive response in the LLNA does not indicate that the compound is a respiratory sensitizer. The definitive classification would involve a deeper understanding of the cellular and molecular events of the sensitization response as well as the cellular and physiological responses during both sensitization and elicitation. Considering the breadth of research, such an approach seems feasible and what may be required is a comprehensive integration of existing endpoints and assessment of their predictive value with a wider and more diverse array of compounds.

One approach may involve examination of the proliferative response in the draining lymph nodes, similar to a LLNA, with complementation with more characterizing endpoints including cytokine profiling, surface marker examination, gene expression analysis and histological assessments. Such endpoints should be mechanistically linked to the development of respiratory allergy which will increase confidence and acceptance of these approaches and may also provide mechanistic insight for the development of more predictive *in vitro* approaches. In addition, endpoints and dosing protocols developed for sensitization approaches should be extended to develop more integrated and consistent elicitation models. Ultimately, the generation of additional data at any level will be invaluable in the development of improved *in silico* approaches for the identification of respiratory allergens (Fig. 2). The final goal of these efforts should be the development of a tiered testing approach to facilitate a simple, accurate and cost-effective assessment of the respiratory allergy potential of a compound. The successful development, acceptance and implementation of such an approach will require effective communication and collaboration between stakeholders from industry, government and academia.

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