

Animal models for protein respiratory sensitizers ☆

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Abstract

Protein induced respiratory hypersensitivity, particularly atopic disease in general, and allergic asthma in particular, has increased dramatically over the last several decades in the US and other industrialized nations as a result of ill-defined changes in living conditions in modern western society. In addition, work-related asthma has become the most frequently diagnosed occupational respiratory illness. Animal models have demonstrated great utility in developing an understanding of the etiology and mechanisms of many diseases. A few models have been developed as predictive models to identify a protein as an allergen or to characterize its potency. Here we describe animal models that have been used to investigate and identify protein respiratory sensitizers. In addition to prototypical experimental design, methods for exposure route, sample collection, and endpoint assessment are described. Some of the most relevant endpoints in assessing the potential for a given protein to induce atopic or allergic asthma respiratory hypersensitivity are the development of cytotoxic antibodies (IgE, IgG1), eosinophil influx into the lung, and airway hyperresponsiveness to the sensitizing protein and/or to non-antigenic stimuli (Mch). The utility of technologies such as PCR and multiplexing assay systems is also described. These models and methods have been used to elucidate the potential for protein sources to induce allergy, identify environmental conditions (pollutants) to impact allergy responsiveness, and establish safe exposure limits. As an example, data are presented from an experiment designed to compare the allergenicity of a fungal biopesticide *Metarhizium anisopliae* (MACA) crude extract with the one of its components, conidia (CON) extract. © 2006 Elsevier Inc. All rights reserved.

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1. Introduction

The incidence of atopic disease in general, and allergic asthma in particular, has increased dramatically over the last several decades in the US and other industrialized nations as a result of ill-defined changes in living conditions in modern western society [1,2]. Because this increase is too dramatic to be explained by genetic changes in the population, it is assumed that changes in environment, lifestyle, and/or medical practices must somehow have contributed to the increase. In addition to the high prevalence of asthma

in the general population, work-related asthma has become the most frequently diagnosed occupational respiratory illness [3]. An estimated 8–20 million workers are exposed to asthmagens in the US. Roughly 90% of these cases involve immunological mechanisms [4] and 60% of those involve responses to proteins [5] as opposed to low molecular weight chemicals as described elsewhere in this issue [6]. Whereas most proteins elicit immune responses, all proteins are not allergenic. Of particular concern are proteins that cause rhinitis and asthma via T helper type 2 (Th2) cell- and IgE-mediated responses. In order to assess the risk of this type of allergenicity associated with inhalation exposure to a variety of proteins, immunotoxicologists have developed animal models of protein allergy. These models have been used both to identify proteins as allergens and to assess the relative potency of various proteins associated with dust mites, molds, and cockroaches in residential environments and detergent enzymes, biotechnology products, and latex in occupational environments. These models also

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have been used to assess the potential of common air pollutants (ozone, diesel, NO₂) to act as adjuvants of respiratory hypersensitivity [7].

A good animal model should exhibit many of the hallmarks of human disease. Respiratory allergy develops in two stages, induction/sensitization and elicitation. During induction, clonal expansion of antigen-specific T and B cells occurs. Under certain conditions that are not fully understood, B cells in genetically predisposed individuals are influenced by Th2 cytokines to undergo immunoglobulin class switching and produce antigen-specific cytophilic antibodies (usually IgE). During antigen challenge, elicitation generally causes a Type I immediate hypersensitivity response by cross-linking antigen-specific IgE molecules bound to mast cells, causing the release of preformed mediators, including histamine and prostaglandins, which are responsible for bronchoconstriction and increased vascular permeability. Between 2 and 8 h after the immediate response, asthmatics experience a more severe and prolonged (late phase) reaction that is characterized by mucus hyper-secretion, bronchoconstriction, airway hyperresponsiveness to a variety of non-specific stimuli (e.g., histamine, methacholine), and airway inflammation characterized by eosinophil influx. This later response is driven by leukotrienes, chemokines and cytokines synthesized by activated mast cells and Th2 cells. Most of the animal models for protein allergy mimic these responses, which provide a number of endpoints that can be assessed including cytophilic antibodies (IgE and IgG1) in serum and lung lavage fluid, inflammatory cells (particularly eosinophils) in lung lavage fluid, induction of antigen-specific lymphocyte proliferation in bronchial lymph nodes, and production of cytokines characteristic of Th2 responses. Whole body plethysmography has also been used to assess both early and late phase airway responses. In humans, these reactions to allergen challenge can lead to chronic inflammation characteristic of chronic asthma [8]. Animal models generally do not mimic this aspect of human asthma.

Animal models of respiratory protein allergy have been developed predominately in mice, rats, and guinea pigs, but occasionally rabbits, monkeys, and dogs are used. Murine models are particularly useful due to the availability of conventionally inbred and transgenic mouse strains which provide the opportunity to ask research questions with regard to sensitive subpopulations (disease susceptibility). Furthermore, a number of mouse strains have been identified as high IgE responders, most notably BALB/c, C57Bl/6, and A/J mice. In addition to the susceptible strains, the availability of murine immunological reagents provides an abundance of tools to assess responsiveness. However, the most important aspects for the use of the mouse model are the similarities between mice and humans in the mechanism of IgE-mediated responses and their antigenic repertoire. Typical models include a sensitizing regimen followed at some interval by antigenic challenge to provoke elicitation responses. The responses measured include respiratory, immune, and inflammatory responses.

Very few of these models have been developed as predictive models to identify a protein as an allergen or to characterize its potency. Here we describe methods we and our colleagues have used in mice (BALB/c) and rats (Brown Norway) to assess allergic responses to mold extracts and house dust mite (HDM), and we present data from a specific experiment designed to compare the allergenicity of a fungal biopesticide *Metarhizium anisopliae* (MACA) crude extract with the one of its component parts, conidia extract (CON). Although we have developed these methods to meet our particular needs to understand the allergenic potential of certain indoor molds and the potential adjuvant effects of air pollutants, the methods we describe are certainly adaptable to study other problems associated with protein allergenicity.

2. Exposure methods

Sensitization can be initiated by either systemic or local exposures. Systemic sensitization can be accomplished by intraperitoneal (IP) injection in the presence of an adjuvant (aluminum-based) as in the classic ovalbumin (OVA) model [9–11], or by cutaneous injections [12] or dermal exposure [13]. Dermal exposures require shaving and/or depilatory removal of the fur, followed in many cases by dermabrasion to remove the stratum corneum prior to application of the agent of interest by ‘painting’ or occlusion [14]. Therefore, IP injections are the simplest and the least time consuming of these systemic exposure methods. Following systemic sensitization, one or more allergen respiratory challenges are required for airway responses to become evident [14]. These methods for respiratory challenge are the same as those used for respiratory sensitization (local organ/site of interest) exposures.

In our laboratory we have systemically sensitized 8-week-old female BALB/c mice to mold extracts by IP injection of 2.5, 25, or 250 µg of MACA (the combination of equal protein concentrations of Hank’s balanced salt solution (HBSS) extracts of *Metarhizium anisopliae* mycelium, spores, and inducible enzymes) in a total volume of 0.2 ml of 1.3% aluminum hydroxide adjuvant (alhydrogel, Superfox Biosector a/s distributed by Accurate Chemical and Scientific Corp., Westbury, NY) [15]. In a similar manner, our colleagues [16] sensitized 8 to 10-week-old female Brown Norway rats strain BN/Ss NHsd) with semi-purified house dust mite (HDM) antigen from *Dermatophagoides fairnae* and *Dermatophagoides pteronyssinus* (Greer Laboratories, Lenoir, NC). The rats were injected subcutaneously with 88 µg HDM suspended in alhydrogel, immediately followed by a 0.5 ml IP injection of *Bordetella pertussis* vaccine (IAF Biovac Inc., Montreal, Canada) containing 1×10^9 heat-killed organisms. Both studies included an adjuvant control group where animals received injections of adjuvant(s) without the antigen.

In both cases, two weeks following systemic sensitization, the animals were challenged by the respiratory route and appropriate endpoints assessed (described below).

Mice were challenged by respiratory aspiration with a maximum of 25 μ g MACA in 50 μ l sterile saline. Rats were challenged by the intratracheal route with 17.6 μ g HDM in 300 μ l sterile saline.

Inhalation exposes the entire respiratory tract. However, whole-body exposures are complicated by the possibility of dermal, ingestion (grooming), and mucosal exposure as well as the need for larger quantities of the agent/protein(s) of interest. Additionally, it is more difficult to assess the dose due to potential differences in respiration rates. Inhalation can also be performed by nose only exposures. This eliminates alternate route exposures but is more stressful to the animals. However, acclimatization to the chambers prior to study initiation can reduce stress.

Intratracheal (IT) exposure insures delivery of precise doses to the lower respiratory tract. However, this procedure requires cannulation of the trachea under anesthesia and considerable skill with the possibility of trauma to the animal.

Intranasal exposures may be performed with or without short-term anesthesia (e.g. isoflurane). The animal is restrained in a supine position with nose pointed upward. The agent is placed at the nares opening and the animal is

allowed to inspire. This method exposes the upper airways while the amount of agent reaching the lower airways is directly proportional to the volume instilled [17]. However, there is the potential for the agent to enter the gastrointestinal tract.

Another method that exposes the lower respiratory tract is aspiration, sometimes identified as intratracheal aspiration. Aspiration also requires anesthesia, but for a shorter time period (with isoflurane anesthesia time $\sim$ 1 min) than intratracheal instillation. In this case, the anesthetized animal is suspended vertically. Using forceps, the tongue is gently pulled out of the mouth in an upward direction, occluding the esophagus. As long as the tongue is held out of the mouth the animal cannot swallow the agent. The agent bolus is then placed in the oropharynx, and the nose is briefly occluded, resulting in aspiration.

Respiratory hypersensitivity is the result of adaptive immune response induction. Therefore, the experimental design must account for the temporal development of the adaptive response. Fig. 1 shows a typical time line used for both a systemic sensitization/respiratory challenge protocol and for a respiratory (local) sensitization. At various times during or following the treatment, samples may be

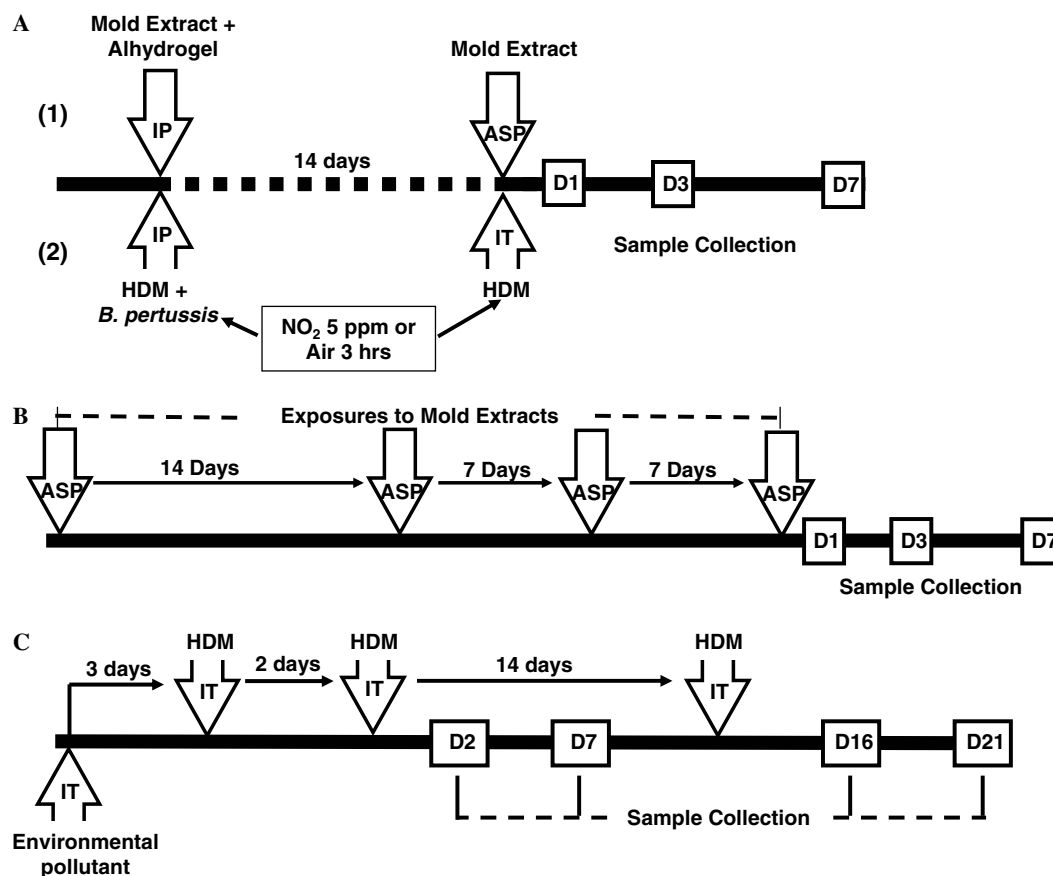


Fig. 1. (A) Systemic sensitization with respiratory challenge protocols have been used [1] to assess the potential of mold extracts to induce allergy in a mouse model [18] and [2] to assess the effects of NO₂ exposure on both allergen (HDM) sensitization and challenge in a rat model [44]. (B) Protocols for respiratory sensitization (local) and challenge to mold extract in the mouse model of mold induced allergy was performed by 4 aspiration (ASP) exposures over a 4 weeks without adjuvant [19]. (C) Local sensitization was used to assess the effects of metals on HDM sensitization [23]. In all cases, samples were collected on various days following the last exposure. IP, intraperitoneal; ASP, respiratory aspiration; IT, intratracheal instillation.

collected to assess the development of endpoints characteristic of allergic asthma or other respiratory hypersensitivity disorders. In our studies of mold-induced allergic airway disease, we have used both systemic sensitization (IP) followed by airway challenge (aspiration) [15,18] (Fig. 1A(1)) and local exposures only (aspiration) [19–21] (Fig. 1B). Overall, using the crude mold extract, responses resulting from the two exposure regimes were similar [19]. However, serum total IgE levels were higher, following the systemic sensitization. Both systemic and respiratory sensitization have been used in assessing effects of air pollutants on allergic responses to HDM (Fig. 1A(2)). To assess the effects of NO₂ exposure on allergic disease, rats were sensitized IP with 100 µg of semi-purified HDM suspended in 0.3 ml saline containing 10⁸ killed *Bordetella pertussis* organisms (Accurate Antibodies, Westbury, NY). Immediately after sensitization, rats were exposed for 3 h to filtered air or 5 ppm NO₂. Fourteen days later, rats were lightly anesthetized by metaflurane inhalation and challenged IT with 50 µg crude allergen suspended in saline, and then immediately exposed to filtered air or 5 ppm NO₂. Respiratory tract sensitization and challenge has also been performed in the rat house dust mite model. The rats were sensitized with 10 µg HDM in 300 µl sterile saline containing in 2 separate IT exposures (under halothane anesthesia) on experimental days –2 and 0 [22,23]. Prior to sensitization, rats were exposed IT to residual oil fly ash or various metals in order to assess the potential adjuvant effects of these air pollutants. Two weeks after the second sensitization, the rats were challenged (under halothane anesthesia) with 10 µg HDM in saline (Fig. 1C).

Another important consideration in the experimental design is the ability to distinguish responses that may be induced by vehicles used to administer the protein(s) of interest, including adjuvants or any other agent that might impact the response outcome. Furthermore, controls that distinguish adaptive immune responses from inflammatory or irritant responses need to be included. For the systemic sensitization studies described above, the controls groups included animals that received injections of adjuvant(s) without the antigen. Additionally, the inflammatory control animals are treated with vehicle during sensitization, but challenged with antigen. Another control that can be included is animals sensitized with antigen, but challenged with vehicle. We have also included exposure to a non-allergenic protein (bovine serum albumin) as a control [20]. Methods for sample collection and the subsequent analysis of these samples are provided below.

3. Sample collection

3.1. Blood collection

To insure that the exposure regime is resulting in the development of an IgE or IgG1 response, it may be important to collect small blood samples (100–200 µl). There are several survival blood collection methods that may be per-

formed once or repeated under certain conditions. A survival blood collection is defined as ≤10% of blood volume (~8% body weight), i.e. 200 µl from a 25 g mouse.

The lateral tail vein is an easily accessible site where blood can be sampled either by using a needle (easier in rats than mice) or by nicking the vein. Nicking is easily performed, relatively non-traumatic, and routinely done without anesthesia, but effective restraint is required. Warming the tail with a heat lamp or warm compress will increase obtainable blood volume. Repeated collection is possible using this method. For one time collection of a very small sample—a single drop of blood, snipping no more than the distal 1 mm of the tail can be done.

The retro-orbital plexus/sinus is another source for a small blood sample from both mice and rats, although it is not the method of choice for rats. A glass capillary is rotated until the sinus is penetrated and blood is collected. The method requires the use of a topical ophthalmic anesthesia or general anesthesia. With a skilled technician, this is a humane procedure and produces minimal and transient pain/distress. However, with an unskilled technician, there is a higher risk of complications. Additionally, blood should not be taken more than once every 2–3 weeks from the same orbit, and care should be taken to insure hemostasis following the procedure.

Another site for blood sampling in both rats and mice is the saphenous vein. Anesthesia is not required, but effective restraint is necessary. With the hind leg extended and fixed by holding the fold of skin between the caudal side of the thigh and tail, the thigh surface is shaved and the visible saphenous vein is punctured using a needle (e.g. 23 g). A drop of blood will appear and can be collected (~100 µl total). Coating the skin surface with silicone grease optimizes blood flow by minimizing blood loss into the surrounding skin. A gauze swab is applied to the puncture site to stop bleeding. Repeated/serial sampling is possible several times in a day by removing the scab. However, this procedure requires more skill than either tail bleeding or retro-orbital sampling, and requires more time due to site shaving. Websites that provide instructions for saphenous vein blood sampling are <http://film.oslovet.veths.no/saphena> and http://www.uib.no/vivariet/mou_blood/Blood_coll_mice_.html.

Larger blood volumes can be collected by intracardiac bleeding (1 ml or more from an adult mouse; 4–8 ml from an adult rat). However, this is considered a non-survival procedure and can only be performed after ensuring that the animal is under deep anesthesia. Another terminal method for collecting larger blood volumes for rats is by abdominal aorta puncture. After insuring surgical plane of anesthesia, the abdominal cavity is cut open, and the internal organs are lifted to expose the descending abdominal aorta (located on the animal's left side). The mesenteric tissue covering the abdominal aorta is separated, and blood can be withdrawn using a Vacutainer or syringe.

Regardless of the collection method, generally the blood is processed to provide either plasma or serum. Blood col-

lected in the presence of an anti-coagulant (e.g., heparin, Alsevers Solution) allows the cells to be separated from the fluid portion, or plasma, by centrifugation. When the collected blood is allowed to clot, the fluid portion is serum.

In our studies, the mice either were treated with mold extracts using one of the treatment protocols shown in Fig. 1, or were immunized by 3 IP injections, the 1st containing adjuvant, 3 weeks apart to develop a high IgE titer. In either case, the collection of large quantities of blood made these non-survival procedures. Therefore, the mice were anesthetized by IP injection of 0.4–0.5 ml of 5 mg sodium pentobarbital/ml saline or 0.2 ml of a 1:10 dilution of euthasol (pentobarbital sodium (390 mg/ml) and phenytoin sodium (50 mg/ml)). Blood samples were collected by cardiac puncture using a 23-gauge 1-in. needle. Similarly in the HDM model, rats were anesthetized with sodium pentobarbital (200 mg/kg) and bled by cardiac puncture. Subsequently, the blood was transferred to a serum separator tube and allowed to clot at room temperature for 1–2 h prior to centrifugation. For long-term storage, the serum was stored at -80°C .

3.2. Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage (BAL) allows assessment of the inflammatory status of the lungs. BAL fluid (BALF) contains cells, cytokines, and antibodies that can be assayed to determine the allergic status of the animal. It is accomplished in lethally anesthetized animals or following sacrifice. The trachea is exposed, subsequent to severing the diaphragm and rib cage, and cannulated (e.g., a blunt 24-gauge needle for mice). The lung lumen is then washed one or more times with saline solutions such as HBSS or phosphate buffered saline (PBS). Some investigators determine lavage volume based on animal body weight while others use a fixed volume from 0.75 to 1 ml. With the lavage solution-filled syringe attached to the cannula, the saline solution is gently pushed into the lungs and aspirated. In our laboratory, mouse lungs are lavaged with two 1 ml aliquots of HBSS that are pooled prior to sample processing. Rats are lavaged 3 times with a single volume of warmed saline (35 ml/kg).

4. Assessment methods

4.1. Airway reactivity assessment

An important feature of allergic asthma is airway reactivity, including both immediate airway responses to specific antigen and airway hyperresponsiveness (AHR), an antigen non-specific response induced experimentally by agents such as cholinergic stimulants (methacholine (Mch)) or histamine. AHR can be assessed by several commonly used methods. Two of these methods, pulmonary function monitoring [18] and whole body plethysmography [20,21] have been used in our laboratory.

Pulmonary function monitoring allows measurement of airway responses in anesthetized animals, but it is a terminal procedure. We have used this procedure to assess airway hyperresponsiveness to a non-specific agonist (methacholine or acetylcholine) [18,16]. There are commercially available systems such as Flexi-vent (SCIREQ Scientific Respiratory Equipment, Inc., Montreal, Quebec, Canada) or Mumed (PMS, Mumed, London, UK), as well as systems built within laboratories. Previously in our laboratory, we used a laboratory built system [18]. The mice were anesthetized with urethane 1.5 g/kg, tracheostomized, and intubated with a T-shaped cannula adapted to measure airway opening pressure and flow. Additionally, spontaneous breathing was blocked by IP injection of succinylcholine chloride. The mice were ventilated with the ventilation rate (controlled by solenoid switching speed) determined for each animal based on allometric scales of body weight vs. lung volumes [24]. Heart rate and waveform were also monitored throughout the experiment (Sinus Rhythm Analyzer, Micro-Med, Louisville, KY). Flow and pressure measurements for each breath were averaged over a 6 s period to calculate the compliance and airway resistance according to the constant flow inflation method [25]. A stable baseline was recorded prior to a Mch challenge with doubling doses (50–400 $\mu\text{g/kg}$ body weight) delivered as a bolus over one second via a jugular cannula connected to an electronically controlled syringe pump. A two minute recovery period was allowed between each dose. Results collected throughout the challenge and recovery periods were expressed as the effective dose (ED) of Mch that caused a 100% increase in airway pressure (ED100) and airway resistance (ED100) over baseline values and a 20% decrease in compliance (ED20). These ED values were compared to normal mice. Airway opening pressure (P_{ao}) was monitored directly and total respiratory system resistance (R_T) and compliance (C_T) were calculated using a custom-written software program described by Gavett et al. [26].

In the HDM model, rats were similarly anesthetized (1.0 g/kg urethane) 24 h after antigen challenge [16]. A transoral cannula was placed in the trachea, and a saline-filled polyethylene 50 catheter was inserted into the jugular vein (for subsequent infusion of saline and then acetylcholine). Rats were then paralyzed with an intramuscular injection of 2.5 mg/kg succinylcholine chloride (Glaxo-Burroughs Wellcome, Research triangle park, NC), placed supine into a whole-body pressure plethysmograph, and ventilated with a small animal respirator at a frequency of 90 breaths/min. An initial baseline measurement of airway pressure was obtained during a period of no infusion followed by intravenous infusion of saline (0.04 ml/min). Rats were then infused with acetylcholine chloride (Sigma chemical Co.) at a dose adjusted for each animal (0.25 mg/g body weight per ml) for 10 min. with a total delivered volume of 2.48 ml. During the infusion, the flow rate of the peristaltic pump doubled every 2 min, starting at 0.04 ml/min and ending at 0.64 ml/min, and airway

pressure was measured with a pressure transducer located distal to the transoral catheter.

We have also assessed AHR, as well as immediate responses to antigen challenge, using whole body plethysmograph (Buxco electronics, Troy, NY). This is a survivable method of airway response measurement that can be performed with either anesthetized or conscious, unrestrained mice or rats. This method allows repeated assessments of AHR to Mch or other non-specific cholinergic stimulants, as well as immediate airway responses to allergen challenge. BioSystem XA software (SFT3812, version 2.0.2.48, Buxco Electronics) was used to calculate enhanced pause (PenH), a unitless parameter, which has been correlated with airway resistance [27]. This calculation is derived from whole body flow parameters (respiratory rate, tidal volume, inspiratory and expiratory times (T_i , T_e), peak inspiratory and expiratory flows (P_{IF} , P_{EF}), and relaxation time (RT)) according to the following equation: $\text{PenH} = ((T_e - RT) \div RT) \times (P_{EF} \div P_{IF})$. However, recent studies have criticized the use of PenH since it is based on ventilation parameters which are not related to increased airway resistance. These studies have shown that increased resistance does not always correlate with increased PenH [28,29].

In our laboratory, AHR to Mch aerosol has been determined by measurement of time-integrated changes in PenH in response to Mch on days 1 and 3 after the final allergen exposure [20,21]. After measurement of baseline PenH for 5 min, either saline or Mch in doubling concentrations (4, 8, 16, 32, and 64 mg/ml) was nebulized and delivered through an inlet of the chamber for 1 min, and measurements of PenH were made for 4, 5, 6, 7 and 11 min., respectively, after each dose. After subtracting baseline values, time-integrated changes in PenH were calculated and expressed as area under the curve (AUC) for each concentration of Mch (PenH units $\times$ s).

Additionally, we have followed the progression of immediate airway responses to allergen sensitization/challenge. Baseline PenH measurements were recorded for 10 min and averaged for each animal. Mice were then exposed by aspiration to fungal extracts or control agents (HBSS), and placed back in the chambers within 5 min of dosing. PenH readings were then monitored and averaged over a 1 h post-instillation period [20,21]. A similar approach was used to assess immediate responses to HDM in rats [30].

One study in our laboratory was designed to compare the responses of mice treated with the *M. anisopliae* combined antigen MACA (an extract of mycelium, conidia, and inducible enzymes) to the component conidia extract (CON) and included bovine serum albumin (BSA, negative control) or HBSS (vehicle control). Eight week old female BALB/c mice were treated with either 10 μ g of MACA, CON, or BSA in 50 μ l of HBSS by aspiration using the treatment protocol shown in Fig. 1B. The vehicle control mice were treated with 50 μ l of HBSS by the schedule. The inflammatory controls exposure regiment was 3 $\times$ to

HBSS and the 4th time to MACA (HB/MA) or CON (HB/CO). Mice were killed and samples collected prior to (D0), day 1 (D1) and 3 (D3) days after the 4th treatment. The assessment for allergic asthma-like responses included both Mch challenge-induced AHR PenH one day after the 4th treatment (data presented as area under the curve) (Fig. 2A) and the development of increased airway responses following each treatment (data presented as percent increase in PenH over baseline values) (Fig. 2B). Mice treated with CON had significantly increased airway responses immediately after the exposures when compared to all other treatments following the 1st and 2nd exposure. Following the 3rd and 4th exposures mice treated with either MACA or CON had significantly increased immediate airway responses compared to all controls. Respiratory hyperreactivity responses to MACA and CON were not significantly different when measured on day 1 by exposure to doubling concentrations of nebulized Mch. Nor were these treatments significantly increased compared to the inflammatory controls with the exception of MACA treated mice following the 64 mg/ml Mch challenge.

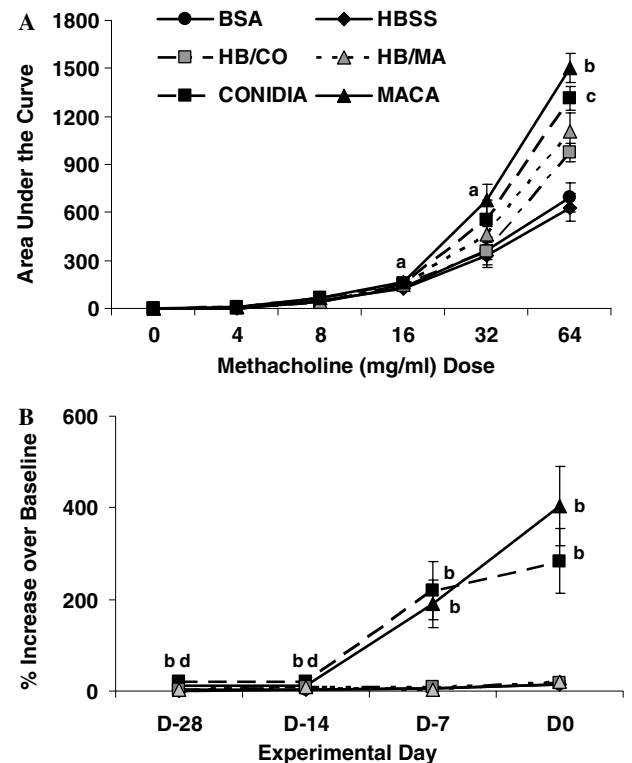


Fig. 2. BALB/c mice were exposed 4 $\times$ by aspiration to *M. anisopliae* extract (MACA), *M. anisopliae* conidia extract (CON), bovine serum albumin (BSA), or vehicle (HBSS). The inflammatory controls (HB/MA, HB/CO) exposure regime was 3 $\times$ to HBSS and the 4th time to MACA or CON. Respiratory responses measured as PenH were assessed (A) to doubling doses of Mch (4–64 mg/ml) (data presented as area under the curve), and (B) immediately after each of the 4 exposures (data presented as % increase over baseline responses). Data shown represent mean $\pm$ SE. $n = 5$; a—different from HBSS at $p < 0.05$; b—different from all controls at $p < 0.05$; c—different from HBSS and BSA at $p < 0.05$; d—CON different from MACA at $p < 0.05$.

4.1.1. *In vitro* tracheal smooth muscle contractions

Another method for assessing airway responses is to measure tracheal tissue smooth muscle contractions in response to cholinergic stimuli (Mch, carbachol) [31,32]. At selected time points following allergen challenge, the trachea is resected, cleaned of extraneous tissue, and sectioned. The tracheal sections are fixed to a strain gauge in an organ bath, an experimental set-up used to investigate the physiology and pharmacology of *in vitro* tissue preparations. The tissue is suspended in a temperature-controlled chamber filled with Krebs-Ringer solution and contractile function is usually recorded with a force (isometric) transducer following an equilibration period. Typically, dose/concentration-response curves are generated from allergen-challenged and naïve/control animal tracheas to cholinergic stimulants.

4.2. BALF cell concentrations

The total cell and differential cell concentrations derived from the BALF provide insight into the quantity and quality of the lung cellular influx following allergen sensitization and challenge. The BALF is centrifuged to separate the cells from the fluid portion. The supernatant is aspirated and stored for biochemical (e.g., total protein, lactate dehydrogenase activity) and immunologically based assays (e.g., antibodies, cytokines). The cell pellet is resuspended in an isotonic solution with a portion used to determine the total cell concentration using one of two methods. In the first method, the cell concentration is determined by microscopic examination using counting chamber or hemocytometer. Additionally, cell viability can be determined simultaneously using trypan blue exclusion staining. The second method determines cell density spectrophotometrically using instruments such as the Coulter Counter (Coulter Corp., Miami, FL). However, with this method cell viability cannot be determined.

Another portion of the cell suspension, usually 100–150 μ l, is adhered to glass slides by centrifugation for differential cell counts. Our laboratory has found mouse cells to be more fragile than rat cells. We have found centrifugation at 200 rpm for 10 min using a Cytospin 2 centrifuge (Shandon Inc., Pittsburgh, PA) provides the best result. Subsequently, Wright-Giemsa staining of the slides allows the cell populations to be identified by differential staining and morphological discrimination. Fig. 3A shows an example of BALF cell slides for (A) mice treated with the vehicle (control) and (B) mice treated 4 $\times$ with 50 μ g of *Penicillium chrysogenum* extract [21].

BALF cell counts, from the study described above, demonstrated that multiple exposures to MACA cause significant increases in total cell counts prior to (D0) and at least 3 days following the 4th IA exposure. However, CON treated mice showed a small but significant increase in total cells on D1 and D3 following the 4th exposure compared to HBSS treated mice by D3. Fig. 3C shows

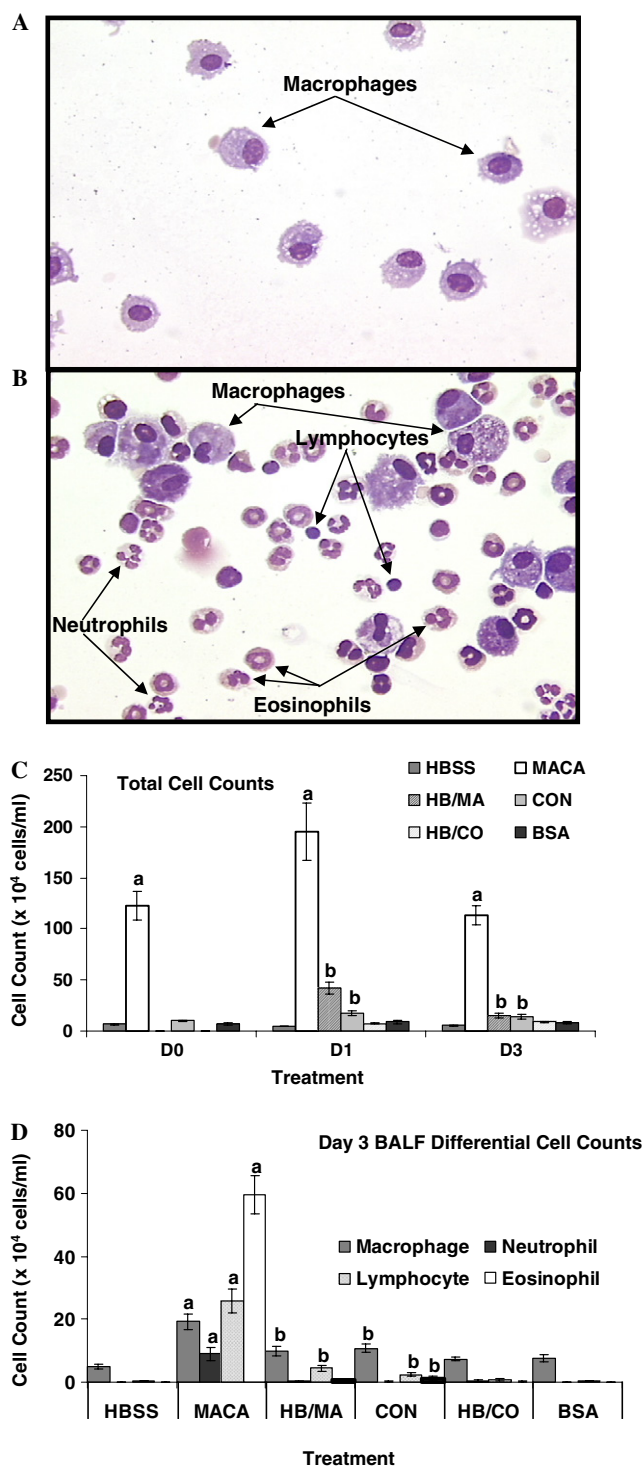


Fig. 3. Bronchoalveolar lavage fluid (BALF) cells were differentially stained with Wright-Giemsa. BALF cells 1 day following 4th treatment from (A) vehicle control mice are relatively low in number (6×10^4 cells) and are predominately macrophages (90.4%). (B) Cells from mice treated 4 $\times$ with 50 μ g of *Penicillium chrysogenum* extract are more diverse with eosinophils predominating (48%) and a significantly elevated total cell count (62.3×10^4 cells) [21]. Forty times of magnification. BALF (C) total cell counts and (D) day 3 (D3) differential cell counts following multiple IA exposures to MACA, CON, or BSA and single exposures to MACA (HB/MA) or CON (HB/CO). Data shown represent mean $\pm$ SE. a—different from all other treatments at $p < 0.05$; b—different from HBSS at $p < 0.05$.

BALF total cells for the three sample collection time points. BALF differential cell counts indicate that multiple exposures to MACA induce a significant increase in all cell types compared to all other treatments. Additionally, a single exposure to MACA (HB/MA) causes a significant increase in neutrophils and lymphocytes compared to HBSS. The neutrophil increase had resolved by D3. CON treatment induced small but significant increases the BALF cells types compared to HBSS. However, the neutrophil increase was resolved by D3. A single exposure to CON (HB/CO) or multiple BSA exposures caused no significant increase in cellular influx. Fig. 3D shows BALF differential cells counts for D3.

4.3. Immunological assessments

The serum, plasma, or BALF from experimental animals can be assayed for proteins of interest in allergy models. These proteins include the cytotoxic antibodies, such as IgE (mice and rats) and IgG1 (guinea pigs and mice), which arm mast cells/basophils for allergic responsiveness. Additionally, these fluids can be assayed for both total antibody isotype concentration and antigen-specific antibodies. Other proteins of interest in allergy models include Th2 relevant and inflammatory cytokines (IL-4, IL-13, IL-5, IL-10, TNF α) and chemokines (Eotaxin, RANTES, IL-8).

In respiratory hypersensitivity models, the enzyme-linked immunosorbent assay (ELISA) is a method for the detection and quantification of response proteins such as antibodies, cytokines, or chemokines. ELISAs are generally described as a capture ELISA or two antibody sandwich ELISA. In the antibody capture ELISA, the antigen of interest is immobilized on a solid phase (microtiter plate) and the antibodies in the sample source (serum, BALF) are allowed to bind. Subsequent, detection is either direct (labeled primary antibody) or indirect (labeled secondary antibody, such as biotinylated anti-IgE). In the two-antibody sandwich ELISA, an antigen-specific antibody is bound to the matrix, followed by binding of the antigen (e.g., antibody isotype such as IgE, cytokines (IL-4, IL-5)). Subsequently, another antigen specific antibody is allowed to bind. The two-antibodies are often referred to as paired antibodies because they both bind the target antigen but do not hinder the binding of the other. Many kits and paired antibodies sets are commercially available particularly for mouse assays. Total serum and BALF IgE ELISA data from the previously described *M. anisopliae* study in our laboratory is shown in Fig. 4. Three days after the final exposure MACA treated mice had significantly elevated levels of total IgE in both serum and BALF compared to all other treatments. CON treated mice had a significant increase only in serum IgE levels compared to HBSS and BSA controls.

Passive cutaneous anaphylaxis (PCA) is another method for detection and quantification of cytotoxic antibodies. Serial dilutions of serum/antibodies from an aller-

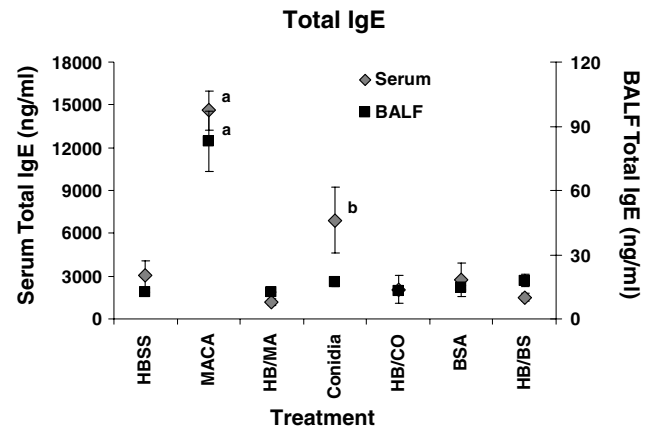


Fig. 4. Serum and BALF total IgE were quantified by ELISA: 3 days after the 4th aspiration exposure to MACA, CON, BSA, vehicle (HBSS) or 1st antigen exposure for the inflammatory controls (HB/MA, HB/CO). Data shown represent mean $\pm$ SE. a—significantly different from all other treatments at $p < 0.05$; b—significantly different from HBSS at $p < 0.05$. $n = 5$.

gen-sensitized animal (guinea pig, rat, and mouse) are intradermally injected into a non-sensitized animal. Subsequent intravenous injection of the allergen mixed with Evans blue dye (a very soluble dye that strongly binds to plasma albumin, and disappears very slowly) results in a characteristic localized evanescent cutaneous reaction (wheal and flare) called the Prausnitz–Kustner reaction. Dye leakage as a consequence of capillary dilatation and increased vascular permeability at the site of antigen-antibody reactions results in a dark blue area (positive response). The IgE (IgG1) antibody titer is determined by the inverse of the highest dilution factor at which the dye is visible. There are two forms of PCA: (1) homologous PCA is when both serum donor and recipient animals are the same species and (2) heterologous PCA is when serum donor and recipient animals are different species. PCA is a sensitive reaction for detecting very small quantities of antibodies.

The rat basophil leukemia-2H3 (RBL) assay system provides an indirect measurement of antigen-specific IgE [33,34]. Rat basophil leukemia cells (RBL-2H3) (ATCC #CRL 2256) grown in 96-well plates are passively sensitized with rat or mouse serum samples. Subsequently, sufficient antigen to provide IgE cross-linking is added to the wells (empirically derived), resulting in mediator release. Measurement of these mediators (histamine and β -hexosaminidase or serotonin from pre-loaded cells) provides an indirect measurement of antigen-specific IgE. In our laboratory, we measure the release of β -hexosaminidase [21]. In addition to spontaneous release and total release, we measure an adjusted spontaneous release in wells treated with mold extracts to account for any extract effects. When the adjusted spontaneous release OD is greater than spontaneous release OD the former is used as spontaneous release in calculating % total release. The optical density is read on a Spectromax 340 PC^R Plate Reader at a wave-

length of 405 nm using Softmax Pro^R version 2.6.1 software for data collection. The RBL-2H3 assay data are expressed as % of total β -hexosaminidase release calculated as follows:

$$\% \text{ Total release} = \frac{\text{Test sample OD} - \text{Spontaneous release OD}}{\text{Total release OD} - \text{Spontaneous release OD}} \times 100.$$

RBL data from the previously described study in our laboratory is shown in Fig. 5. The addition of MACA to the passively sensitized RBL cells resulted in a significant release of β -hexosaminidase only when the serum used to sensitize the cells came from mice that had received multiple exposures to MACA.

4.3.1. Multiplex assays

More recently, instruments such as Luminex (Luminex Corporation, Austin, TX) have been developed to use a relatively small sample volume to assay for multiple cytokines or chemokines, as well as other proteins, in a 96-well plate format. One type of assay system is similar to a sandwich ELISA in that paired antibodies are used to detect proteins of interest in BALF or serum samples. However, unlike ELISAs, multiple analytes can be detected simultaneously. Polystyrene beads are loaded with red and infrared fluorophores at different ratios. Therefore, each of the 100 bead sets has a unique spectral signature and is designed to assess one analyte. One of the paired antibodies is bound to the bead surface. Following incubation with the sample fluid and the orange fluorescing detection antibody, the bead stream is drawn up past the two lasers to identify the bead set (red laser) and to quantify the fluorescence of the detecting antibody (green laser). This multiplexing enables the quantification of a number of endpoints in what are often limited samples such as mouse serum or BALF.

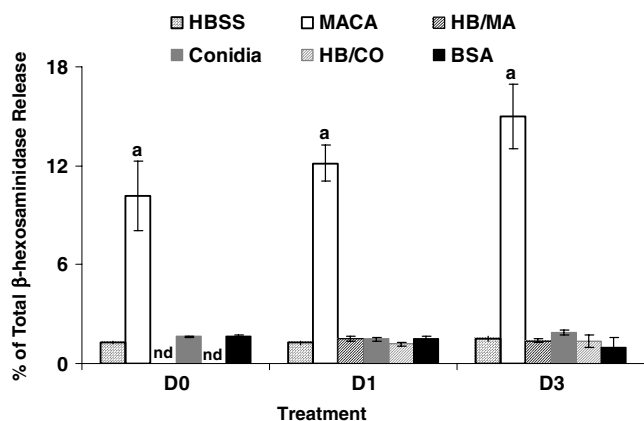


Fig. 5. RBL assay: D0 (before) and D1 and D3 after the 4th aspiration exposure to MACA, CON, BSA, vehicle (HBSS) or 1st antigen exposure for the inflammatory controls (HB/MA, HB/CO). Data shown represent mean $\pm$ SE. a—significantly different from all other treatments at $p < 0.05$. $n = 5$.

4.4. Messenger RNA (mRNA)

4.4.1. Polymerase chain reaction (PCR)

It is not always possible to identify proteins (cytokines, chemokines) of interest when species specific reagents are more limited, as they are for rats and Guinea pigs, or when newly identified protein endpoints are being developed. In these cases, mRNA from tissue homogenates of lymph nodes, spleen, or lungs can be amplified and quantified by PCR. This method may be particularly informative when comparing allergen challenged to naïve/control animal tissues. It is important to acknowledge that the presence of, or an increase or decrease in, mRNA for a particular end point while suggestive does not always equate to resulting protein.

PCR is a three step process performed in an automated cyclor that can heat and cool the reaction mixture tubes in a short time frame. These cycles are repeated 30–40 times and result in the exponential increase in the number of gene copies due to the copying of both DNA strands. First, the double stranded DNA is denatured at 94 °C to form two single strands of DNA. Additionally, enzymatic reactions from the previous cycle stop. Secondly, the primers are allowed to anneal at 54 °C. Each primer is designed to be complementary to one of the template DNA strands and border the DNA of interest. The strength of the bond formed depends upon the goodness of fit between the primer and the template. This bond allows time for the Taq DNA polymerase to attach to the newly formed double stranded DNA (template and primer) and to begin copying the template. The addition of each base increases the strength of the bond between the template and the primer, decreasing the chance of disruption of the template-primer pair. For the third step, the temperature is increased to 72 °C, the ideal working temperature for the polymerase, allowing extension of the cDNA where primers are strongly bound. The primers that fit perfectly with the template and have had a few bases added have a stronger bond to the template than the forces breaking these bonds. However, the increased temperature results in the release of primers that have bound weakly due to a poor fit. Thus extension of the template complement primarily occurs where the primers fit exactly.

A standard PCR mix (25 μ l) consists of 100 ng DNA template (genomic DNA or cDNA produced from mRNA template, 1 $\times$ PCR buffer (500 mM KCl; 100 mM Tris-HCl (pH 8.3); 15 mM $MgCl_2$ (Perkin Elmer Cetus recommendations)), dNTPs mix (200 μ M each nucleotide), 0.4 μ M of each primer, 1 unit Taq DNA polymerase and autoclaved ultra-pure water (pH 7.0). Before using the PCR product in other applications, it should be checked to insure that a product was formed, the product is the expected size, and that only one band is formed. Lack of a product can result from poor quality template DNA, a primer that does not fit, or too much starting template. An unexpected size or number of product(s) may result when a primer fits on more than one gene site or both primers fit on a different gene.

4.5. Lung histopathology

Although BALF assessments provide insight into the status of the animal lung, fixed lung sections provide direct evidence of lung pathology. Fixed lungs (lung sections) are embedded in paraffin, and sectioned. For our laboratory, the lung lobe is sectioned along the main stem bronchus and stained with hematoxylin–eosin solution prior to pathological examination. The relative degree of severity of inflammatory, degenerative, and proliferative changes was graded on a scale of one to five: 1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately severe, 5 = severe/high. The pathology scores for lung sections are summarized as follows: *incidence* is the number of mice exhibiting a particular pathology; *severity* is the numeric average of the pathology scores for a particular lesion per treatment group.

In addition to pathological examination, fixed lung slide preparations can be stained immunohistochemically to identify the presence and possibly the cellular source or localization of molecules of interest. In our studies, we have demonstrated that certain neurotrophins (NGF, NT-3, NT-4) are elevated in the BALF of mold extract exposed mouse as they are in human asthmatics. Subsequently, immunohistochemical analysis indicated that mononuclear cells and epithelial cells are the source of NGF [35].

5. Safety/risk assessment

Animal models have a role not only in the elucidation of mechanisms of disease pathology, but also in hazard identification and in setting occupational exposure limits. Our laboratory has used a BALB/c mouse model to evaluate the allergic potential of molds that have been identified in water-damaged homes and building (*Stachybotrys chartarum* [20], *Penicillium chrysogenum* [21], or that may be used for indoor pest control (*M. anisopliae* [15,18,19]. This model has demonstrated that these fungal extracts can induce antigen non-specific lung injury through increased permeability (bronchoalveolar lavage fluid (BALF) total protein) and cell damage (BALF lactate dehydrogenase (LDH) activity), as well as neutrophil influx. These non-specific responses appeared shortly after exposure and waned by 3 days post exposure. Additionally, multiple exposures to these extracts induced responses indicative of an allergen-specific immune response, including elevated BALF IL-5 and eosinophils, serum total and antigen-specific IgE, and immediate airway reactivity following fungal extract challenge, and AHR to Mch challenge. Furthermore, the data presented here suggests that the various mold life stages or components (hyphae, spores/conidia, inducible proteases/chitinases) may result in different responses. Multiple respiratory exposures to CON induced significantly elevated BALF cell counts (total and differential), serum IgE and airway responses compared to HBSS (vehicle control). However, these responses with the exception of airway responses were significantly lower than those induced by

MACA the combined component extract. These data strongly suggest that the use of an animal model to assess a mold's potential to induce respiratory hypersensitivity should not be limited to one component/live stage of the mold. The conclusion from this work is that *S. chartarum*, *P. chrysogenum*, and *M. anisopliae* have the ability to induce responses in mice that are characteristic of human allergic asthma. The *P. chrysogenum* extract was shown to induce these responses in a dose-dependent manner. However, a higher dose of *P. chrysogenum* extract was required to obtain a level of responsiveness similar to that demonstrated in *M. anisopliae* extract-exposed mice. These results indicate that *P. chrysogenum* may be less potent at allergy induction. Recently, we have identified a *M. anisopliae* mycelium catalase as an allergen in the mouse model using immunoblots and mass spectrometric analysis [36]. Additionally, the mouse sera developed against the *M. anisopliae* extract was shown to be cross-reactive not only with catalase from other sources, but also with proteins in mold mixes used in human skin prick testing.

Of particular interest and importance, the detergent industry has demonstrated that animal models can be used to set safe human exposure levels for detergent enzymes [37,38]. In 1969, Flindt [39] and Pepys et al. [40] reported adverse health effects in British workers exposed to a powdered detergent enzyme, *Bacillus subtilis* subtilisin A (alcalase) in 1967. Similar effects were reported in US workers exposed prior to the publication of the British findings [41]. From 1968 to 69, 40 to 50% of the plant population became sensitized with exposures estimated at 30–300 ng/m³. A variety of mechanical reforms were instituted in the detergent industry. Specific to Proctor and Gamble, there have been no new cases of occupational asthma since 1994 [42]. Furthermore, with the exposure level for alcalase set at <15 ng/m³, <20% of the plant population have been sensitized to enzymes in a 10 year period [42]. Animal models have been used to assess the relative potency of new detergent enzymes compared to alcalase (occupational exposure guideline (OEG) level of 15 ng/m³), and exposure levels for the new enzymes have been set accordingly. In this case, the animal model data has been predictive of human health outcomes and has provided a method to ensure the protection of this industrial work force.

6. Summary

The methods and models described above have been applied in a number of studies in our laboratories. The HDM model has been used to elucidate mechanisms associated with allergic disease [16,30] and to demonstrate adjuvant effects of air pollutants such as NO₂ [43], residual oil fly ash and its metal components [22,23], as well as attenuating effects of feed restriction and dioxin treatment [44,45]. The mold allergy model has been used to demonstrate the allergenic potential of extracts from several mold species and to identify the allergenic component in one of these mold extracts [15,20,21,36]. We have been able to demon-

strate dose responses with the mold extract, suggesting that this model has the potential to assess relative potency of different indoor allergens. In addition, we have shown that there are proteins that do not elicit responses when applied in this model [20]. Although these methods and models have been developed to address our specific research needs, they could be applied to other allergens and adjuvants and could be used to address a number of issues related to environmental exposures and allergic lung disease.

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