

A comparison of the allergic responses induced by *Penicillium chrysogenum* and house dust mite extracts in a mouse model

Abstract A report by the Institute of Medicine suggested that more research is needed to better understand mold effects on allergic disease, particularly asthma development. We compared the ability of the fungal *Penicillium chrysogenum* (PCE) and house dust mite (HDM) extracts to induce allergic responses in BALB/c mice. The extracts were administered by intratracheal aspiration (IA) at several doses (0, 2.5, 5, 10, 20, 40, and 80 μ g) four times over a 4-week period. Three days after the last IA exposure, serum and bronchoalveolar lavage fluid (BALF) were collected. The relative allergenicity of the extracts was evaluated based on the lowest dose able to induce a significant response compared to control (0 μ g) and the robustness of the response. PCE induced the most robust response at the lowest dose for most endpoints examined: BALF total, macrophage, neutrophil, and eosinophil cell counts, and antigen-specific IgE. Taken together, our data suggest that PCE may induce a more robust allergic and inflammatory response at lower doses than HDM.

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Practical Implications

Our data suggest that *Penicillium chrysogenum* is a robust allergen and may be a more potent allergen source than house dust mite (HDM) in this mouse model. Two critical factors in the development of human allergic disease, exposure levels and sensitization thresholds, are unknown for most allergens including molds/fungi. Human exposure levels are not within the scope of this article. However, the data presented suggest a threshold dose for the induction of allergic responsiveness to *P. chrysogenum*. Additionally, *P. chrysogenum* as well as other molds may play an important role in asthma development in our society.

Introduction

Molds are ubiquitous in the environment and exposures to molds contribute to various human diseases including allergic lung diseases. The Institute of Medicine reports (NAS 2000, 2004) and World Health Organization (WHO) guidelines (2009) concluded that the role of molds in asthma induction is not clear but an association exists between damp buildings, the presence of mold and asthma exacerbation. Mudarri and Fisk (2007) estimated that 21% of current asthma in the US may be attributed to dampness and molds. A review by Ledford (1994) determined that *Aspergillus*, *Cladosporium*, and *Penicillium* were the most

prevalent fungal genera associated with mold allergy. Some epidemiological studies have shown an association between *Penicillium* and asthma (Eggleston et al., 1998; Chou et al., 2003) or asthma symptoms (Turyk et al., 2006). Additionally, dampness, which promotes fungal growth, is often linked to building-related illnesses (Kildeso et al., 2003) and a review by Denning et al. (2006) described epidemiological evidence of an association between fungal exposure and asthma severity. Cooley et al. (1998) found a strong association between the presence of both *Penicillium* species (especially *P. chrysogenum*) and *Stachybotrys* species and sick building syndrome. Trout et al. (2001) also found an association between *P. chrysogenum* and

building-related illness. However, such species-specific associations generally are not made or are complicated by positive results to multiple molds and/or other agents.

The genus *Penicillium*, consisting of approximately 250–300 species, includes species that grow in xerophilic to hydrophilic conditions and some can produce a number of mycotoxins. One of the species commonly found indoors including in water-damaged homes and buildings is *P. chrysogenum*. *Penicillium chrysogenum*, previously known as *Penicillium notatum* (Samson et al., 1977), grows best in high water conditions but tolerates low water conditions. Furthermore, Wilson et al. (2004) determined that *P. chrysogenum* spores from ceiling tiles stored at 25°C, 20–60% relative humidity (conditions similar to those found in homes) were culturable after 8 months. Although *P. chrysogenum* is used commercially to produce penicillin, xanthocillin X and other secondary metabolites/enzymes, it is generally considered a contaminant. However, *Penicillium* species can cause infections in immune-compromised individuals.

Allergy, a Type I or immediate-type hypersensitivity reaction, is an immune response to otherwise innocuous ‘non-self’ agents (generally proteins) in genetically predisposed individuals. Although most proteins are capable of eliciting immune responses, not all proteins are allergens (Viana et al., 2002). Generally, there are no apparent disease symptoms during allergy induction (sensitization). However, some B cells switch production of immunoglobulin (Ig) isotype from IgM to IgE (or IgG1 in guinea pigs) antibodies. In previously sensitized individuals, allergen re-exposure/challenge cross-links antigen-specific mast cell/basophil-bound IgE antibodies resulting in the immediate release of preformed mediators (including histamine and prostaglandins). Disease symptoms become apparent as these mediators produce bronchoconstriction, increased vascular permeability and inflammation. The clinical manifestations of allergy range from skin rashes and rhinitis to life-threatening asthmatic and anaphylactic reactions. In the case of asthma, a late-phase response may also be seen between 2 and 12 h after challenge characterized by mucus hypersecretion, bronchoconstriction, airway hyper-responsiveness (AHR) to non-specific stimuli, e.g. histamine or methacholine (Mch), and eosinophilic airway inflammation. Some late-phase and chronic components are cell-mediated and categorized as Type IV hypersensitivity responses.

Three *P. chrysogenum* proteins have been identified as allergens using asthmatic sera to demonstrate IgE-reactivity: Pen ch 13 (34 kD), Pen ch 18 (32 kD), and a β -N-acetylglucosaminidase homologous protein (68 kD) (Shen et al., 1995, 1999, 2003; Chou et al., 2002). Shen et al. (1999) found that 24% of 70 asthmatic sera were IgE reactive with *P. notatum* (now *P. chrysogenum*) proteins. Of the IgE-reactive

proteins, there was an 80% binding frequency to the 32 and 34 kD proteins. These proteins, Pen ch 13 (34 kD) and Pen ch 18 (32 kD), have been identified as an alkaline and vacuolar serine protease, respectively (Shen et al., 1999, 2003; Chou et al., 2002).

Animal studies with *P. chrysogenum* have not only demonstrated its ability to induce allergic and asthma-like responses but have also suggested the responses were induced in a dose-dependent manner (Cooley et al., 2000; Schwab et al., 2003; Chung et al., 2005). However, mold studies addressing the issue of relative allergenicity are limited. Relative allergenicity studies provide comparisons to better characterized agents and thus insight into unhealthy environments.

The study goal was to compare the allergic responses induced by the mold *P. chrysogenum* (PCE) extract and HDM, a known inducer of allergic asthma (NAS 2000), in an allergy mouse model.

Materials and methods

Animals

Fifty-day-old female BALB/c mice (Charles River, Raleigh, NC, USA) were group-housed in polycarbonate cages with hardwood chip bedding in an environmentally controlled, Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited vivarium. Mice were maintained on a 12-h light/dark cycle and allowed access to food (Purina Rodent Lab Chow, St. Louis, MO, USA) and water *ad libitum*. Mice were allowed to acclimate at least 1 week prior to the start of the experiment. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of NHEERL, US EPA.

Fungal antigen preparation

The *P. chrysogenum* (ATCC MYA-4644), isolated from bronchoalveolar lavage fluid (BALF) from a patient in Ohio, was grown on a nylon filter overlay of potato dextrose agar at 23°C for 6 weeks as previously described (Vesper et al. 1999). The *P. chrysogenum* extract (PCE; mycelium and spores) was prepared by a modification of the methods described in Ward et al. (1998). Briefly, the fungal mass was extracted by grinding with a sterile mortar and pestle for approximately 5 min followed by Polytron (Kinematica, Basel, Switzerland) homogenization for 2 min in a total of 15 volumes (by weight) of Hanks’ balanced salt solution (HBSS; Gibco BRL, Life Technologies Inc., Rockville, MD, USA) + 0.05% (v/v) Tween 80 (Fisher Scientific, Pittsburgh, PA, USA). The resulting suspension was stirred overnight at 4°C, and then centrifuged at 12,500 g for 1 h at 4°C. The supernatant was decanted and then adjusted to pH 6.0 with HCl. This

filtrate was concentrated with a stirred-cell concentrator (Amicon, Inc., Beverly, MA, USA) using YM3 membranes (molecular weight > 3000 Da cutoff) and filter sterilized with a 0.2- μ m syringe filter. This crude antigen extract was assayed for total protein concentration using a Pierce BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard according to manufacturer's procedures. PCE was stored at -80°C until use. Endotoxin level of the extract was measured using a Limulus amoebocyte lysate test kit (BioWhittaker, Walkersville, MD, USA); the level at the highest dose (80 μ g protein) was 0.10 EU (Endotoxin Unit).

Lyophilized whole body extracts of HDM *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* purchased from Greer Laboratories (Lenoir, NC, USA) were rehydrated in HBSS to 3 mg/ml, mixed equally and stored in aliquots at -80°C until use. Endotoxin level of the highest dose (80 μ g) was 0.27 EU.

Experimental design

Dose-response study. Six mice/dose were exposed to 0, 2.5, 5, 10, 20, 40, or 80 μ g of extract in HBSS (total volume of 50 μ l) by intratracheal aspiration (IA) four times over a 4-week period, as previously described (Ward et al., 2000). Briefly, mice were anaesthetized by inhalation of a mixture of 3% isoflurane and 97% oxygen. Antigen extract was deposited into the oropharynx, after which mice inhaled the extract when their noses were gently occluded with a fingertip. The rationale for the exposure protocol (Figure 1) is based

on the immunology paradigm that the primary immune response is approximately 7–14 days following the initial exposure (Day-28 to Day-14) and approximates the allergy induction sensitizing phase. The second exposure in our protocol is 14 days (D-14) after the first and thus is on the boundary between sensitization and challenge. However, the last two exposures (D-7 and D0) are challenge exposures. Serum and BALF were collected 3 days following the final exposure. BALF was assessed for lactate dehydrogenase (LDH), total protein, total cell and differential cell counts. Serum was assessed for total and PCE or dust mite-specific IgE.

Single dose study. This study was designed for suboptimal exposures. However, the data provides a comparison of the cellular influx and methacholine challenge airway responses. The mice received either 1 or 3 exposures IA to the extracts (Figure 1B). A 20 μ g exposure dose was chosen as the dose where all extract-induced responses were significantly increased compared to the 0- μ g dose. Airway responsiveness was assessed 1 day after the final exposure using a whole body plethysmograph (see Methods below), and BALF was collected 2 days after the final exposure for total and differential cells counts.

Airway responsiveness to methacholine aerosol

As previously described by Viana et al. (2002), airway responsiveness to methacholine (Mch) aerosol was measured in unrestrained mice using whole body

(A) Experimental timeline

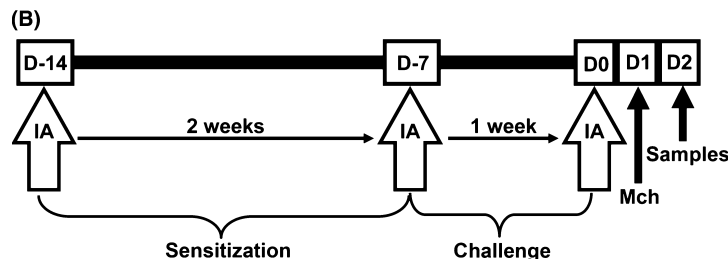
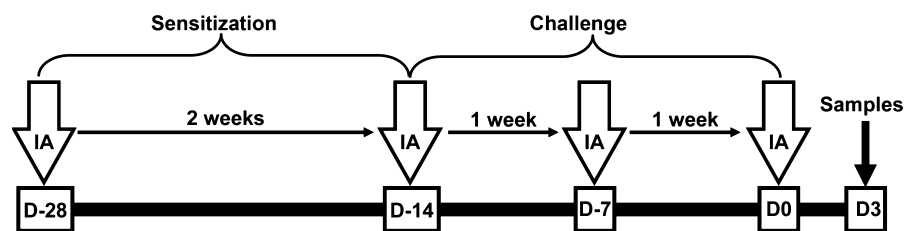


Fig. 1 Experimental time line. (A) In the dose-response study, mice were administered PCE, house dust mite (HDM), or Hanks' balanced salt solution (HBSS) (control group) by intratracheal aspiration (IA) four times over a 4-week period. Allergy sensitization and challenge phases are indicated. Blood and bronchoalveolar lavage fluid (BALF) were collected 3 days after the final exposure. (B) In the single-dose study, mice were administered PCE, HDM, or HBSS by IA three times over a 3-week period. Mch challenge was performed on day 1 and blood and BALF were collected on day 2 after the final exposure

plethysmography (Buxco Electronics, Troy, NY, USA) as time-integrated changes in enhanced pause (PenH, a unitless index of airway hyper-reactivity) 1 day after the final IA exposure. After measurement of baseline PenH for 5 min, either saline or Mch (6.25, 12.5, and 25 mg/ml sequential doses) was nebulized and delivered through an inlet of the chamber for 1 min, and measurements of PenH were made for 10 min after each nebulization. After subtracting baseline values, time-integrated changes in PenH were calculated [Bio-System XA software (SFT3812, version 2.0.2.48; Buxco Electronics)] and expressed as area under the curve (AUC) for each concentration of Mch (PenH $\times$ sec). Methacholine responsiveness was analyzed across treatment groups and within individual mice using SAS software (SAS Inc., Cary, NC, USA). The data are presented as area under the curve.

Bronchoalveolar lavage (BAL) and blood collection

BALF samples were collected as previously described in (Ward et al., 1998). Briefly, mice were anaesthetized with Euthasol (VIRBAC Corporation, Fort Worth, TX, USA) and blood samples were collected by cardiac puncture. The blood was allowed to clot for 1–2 h at room temperature prior to serum separation by centrifugation (Eppendorf 5810 R; Brinkman Instruments, Westbury, NY, USA), and the serum was stored at -80°C until analysis. The lungs were lavaged twice with 1 ml aliquots of room temperature HBSS, and the pooled BALF aliquots were stored on ice. The BALF was centrifuged at 100 g for 15 min at 4°C (IEC/Centra MP4R). An aliquot of the supernatant was stored at 4°C for total protein and LDH activity assays and the remainder stored at -80°C . The cell pellet was resuspended in 1 ml of HBSS. Total BALF cell counts were obtained using a Coulter counter (Coulter Corp., Miami, FL, USA). Additionally, 100–150 μl of resuspended cells was adhered onto glass slides at 200 rpm ($4.3 \times$ gravity) for 10 min using a Cytospin 2 centrifuge (Shandon Inc., Pittsburgh, PA, USA). The BALF cells on glass slides were stained with Wright-Giemsa (Fisher Scientific) using a Hema-Tek 2000 slide stainer (Miles, Inc., Elkhart, IN, USA) and were differentially counted at 200 cells per slide (one slide per animal).

Total protein and lactate dehydrogenase (LDH) assays

BALF samples were assayed for total protein using Pierce Coomassie Plus Protein Assay Reagent (Pierce). Concentrations were determined from a standard curve using BSA standards obtained from Sigma Chemical Co. (St. Louis, MO, USA). Additionally, the BALF samples were assayed for LDH activity using a commercially prepared kit and controls from Sigma Chemical Co. The assays were modified for use on the KONELAB 30 clinical chemistry spectrophotometer

analyzer (Thermo Clinical Labsystems, Espoo, Finland).

Total IgE ELISA

All reagents and incubations were at room temperature and all volumes added were 100 μl unless otherwise noted. Total IgE ELISA was performed as previously described by Ward et al. (1998). Purified monoclonal anti-mouse IgE (PharMingen, San Diego, CA, USA) in phosphate-buffered saline, pH 7.3, was used as the capture antibody and biotinylated rat anti-mouse IgE (BD Bioscience PharMingen) as the detection antibody. Streptavidin-conjugated horseradish peroxidase (Zymed, San Francisco, CA, USA) was the detection enzyme with tetramethylbenzidine (TMB) substrate (DAKO Corporation, Carpinteria, CA, USA). Optical density was read on a SpectraMax 340PC Plate Reader (Molecular Devices Corp., Menlo Park, CA, USA) at a wavelength of 650 nm. Softmax Pro[®] version 2.6.1 (Molecular Devices Corp.) software was used for data collection and conversion from optical density to protein concentrations. The limit of detection for this assay was 6.25 ng/ml.

Antigen-specific IgE assay

A rat basophilic leukemia (RBL) cell β -hexosaminidase release assay was performed as an indirect measure of antigen-specific IgE in serum. The procedure is based on the method by Hoffmann et al. (1997), with modifications by D. Leadbeater and D. A. Basketter (personal communication, Unilever Safety and Environmental Assurance Center, UK). The procedures have been previously described in Chung et al. (2005). Briefly, 96-well flat bottom tissue culture plates were seeded with 10^5 RBL-2H3 cells (ATCC, Rockville, MD, USA) and incubated for 18 h at 37°C in a 5% CO_2 humidified incubator. The cells were passively sensitized with a 1:4 dilution of individual mouse serum or with normal sera (spontaneous and total release controls) for 2 h. Cells were then washed with Tyrode's buffer followed by the addition of allergen extracts (10 μg) and incubated for 1 h at 37°C in a 5% CO_2 humidified incubator. For spontaneous release, cells were incubated with buffer alone. β -hexosaminidase, the mediator measured, is a chitinase and chitinases are produced by some fungi. Therefore, an additional control was used to identify extract-specific (endogenous) chitinase activity; both PCE and HDM extracts were added to cells that were not passively sensitized (no serum). The higher OD for spontaneous release or extract-specific release was used to calculate the % of total mediator release. For total release, cells were incubated with 1% (v/v) Triton X-100 (Sigma) in Tyrode's buffer. Subsequently, the β -hexosaminidase activity was measured by combining cell culture

supernatant and substrate (p-nitrophenyl *N*-acetyl β -D-glucosaminide) in a sterile 96-well plate and incubating the plate for 1 h at room temperature. The enzyme reaction was stopped by the addition of 0.2 M glycine. Absorbance was measured 30 min later using a SpectraMax 340 PC Plate Reader (Molecular Devices Corp.) at a wavelength of 405 nm and data collected by Softmax Pro[®] software (version 2.6.1; Molecular Devices Corp.). Data are presented as percent total release following subtraction of spontaneous release or extract-specific activity if higher. The extract dose resulting in 10% of total release was calculated by interpolation.

interactive effects. Subsequently, exposure-dose groups were compared to the control values, using a Dunnett's test. This was performed to examine differences from the control group without reference to the ANOVA findings. The level of significance was set at 0.05.

Additionally, the estimated dose at which 10% of total mediator release occurred was calculated for the antigen-specific IgE (RBL assay) data. This was accomplished through linear interpolation between responses at consecutive doses bracketing the 10% value. The level of significance was set at 0.05.

$$\% \text{ of Total } \beta\text{-hexosaminidase release} = \frac{(\text{Sample release OD} - \text{spontaneous release OD or extract-specific release OD})}{\text{Total release OD} - \text{spontaneous release OD}} \times 100$$

Endogenous chitinase activity was measured for both HDM and PCE by adding chitinase substrate to 0, 1, 5, and 10 $\mu\text{g/ml}$ of each extract, as well as, 10 $\mu\text{g/ml}$ of trypsin and processing as described above.

Protease assay

Extracts were analyzed for protease activity using the EnzChek7Protease Assay kit (red fluorescence) (Molecular Probes, Eugene, OR, USA). Triplicates of five concentrations (0–20 $\mu\text{g/ml}$) of the extracts as well as a trypsin enzyme control were added to the digestion buffer (200 mM Tris-HCl, pH 7.8, containing 2 mM sodium azide). Following the addition of BODIPY7 TR-X labeled casein substrate in 0.1 M sodium bicarbonate, pH 8.3, the samples were protected from light and incubated for 1 h. Protease-catalyzed hydrolysis of the substrate and subsequent release of BODIPY7 TR-X dye-labeled peptides results in increased fluorescence that is proportional to protease activity. Fluorescence of the BODIPY7 TR-X dye was read in a fluorometer (Spectra Max, Gemini XS; Molecular Devices) using a filter with excitation = 590 ± 10 nm, emission = 645 ± 20 nm. Data are reported as fluorescent units.

Statistical analysis

The data were analyzed in a two stages. A two-way analysis of variance (ANOVA) model was applied to the data, without the control group. This allows the examination of the main effects, of exposures (HDM and *P. chrysogenum*) and doses (2.5, 5, 10, 20, 40, 80 μg extract protein), and

Results

Total protein and LDH levels

Elevations in BALF total protein are indicative of pulmonary vascular leakage (pulmonary edema). Mice exposed to 2.5 μg of PCE or 10 μg of HDM showed a significant increase in total protein levels compared to the 0- μg dose control mice (Figure 2A). The response to HDM extract displayed a trend toward dose-dependent increases across the dose range while the response to PCE appeared to plateau at the 20- μg dose. Additionally, PCE dose above 10 μg did not significantly increase the level of BALF total protein within the treatment group. Exposure to 2.5–20 μg of PCE caused a significant increase in BALF total protein compared to HDM within each dose group.

Elevated LDH activity in BALF is indicative of non-specific cellular damage. Mice exposed to 20 μg of either extract showed a significant increase in LDH activity compared to the 0- μg dose controls (Figure 2B). However, the LDH activity level continued to increase in mice exposed up to 40 μg of HDM and declined in mice exposed to PCE at 40 and 80 μg .

BALF total and differential cell counts

BALF total and differential cell counts were assessed to determine the relative effect of the extracts in the development of the late-phase allergic response in this mouse model. Generally, BALF cellular influx response to HDM increased across the dose range. Although the response to PCE was significantly elevated at lower doses than HDM, the response to

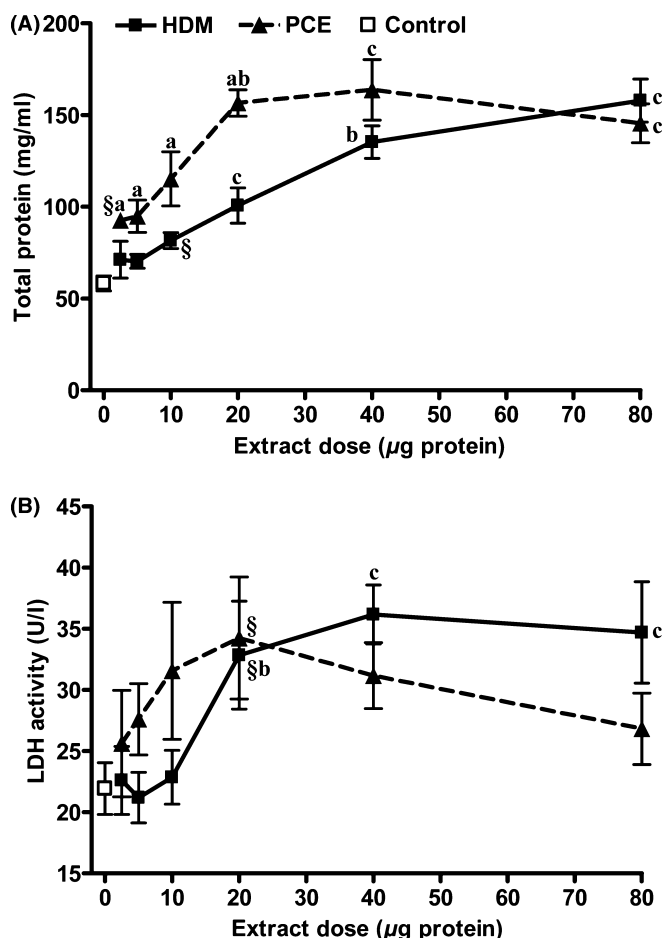


Fig. 2 Total protein (A) and lactate dehydrogenase activity (B) in bronchoalveolar lavage fluid of house dust mite (HDM)- or PCE-exposed mice collected 3 days after the final exposure. Symbols indicate (§) the lowest significant dose compared to Hanks' balanced salt solution (0 dose control), statistically significant differences (a) between extracts at the same dose, (b) from all lower doses within treatment, (c) from 2.5-µg and 5-µg doses within treatment ($P < 0.05$). Error bars represent standard error of the mean. Control $n = 12$; HDM and PCE $n = 5-6$.

PCE generally reached a plateau at approximately the 10–20 µg dose range. The lowest doses of HDM and PCE that induced a significant increase in total cell, macrophage, lymphocyte, and eosinophil counts compared to 0 µg dose controls were 5 and 2.5 µg, respectively (Figure 3A–D). However, to significantly increase the neutrophil counts compared to the control mice required 20 µg of HDM and 5 µg of PCE (Figure 3E). Additionally, PCE-exposed mice showed a significant elevation compared to similarly dosed HDM-exposed mice in total cells, lymphocytes, and neutrophils from 2.5 to 40 µg doses and macrophages from 2.5 to 20 µg doses and eosinophils from 5 to 20 µg doses. These data suggest that low-dose exposure to PCE carries an increased risk regarding the influx of inflammatory cells compared to HDM.

In the single dose study, mice received either 3 or a single exposure to 20 µg HDM, PCE, or HBSS with BALF collected 2 days after the final exposure. With a single exposure, HDM induced a larger influx of BALF total cells as well as neutrophils and eosinophils compared to PCE (Table 1). The level of inflammatory cell influx following three 20-µg doses indicates that multiple exposures do not significantly elevate neutrophil levels over those observed with a single dose of either extract. In contrast, multiple exposures to either of these extracts clearly induce a more robust eosinophil influx (11.5-fold increase for HDM to 50.2-fold increase for PCE) (Table 1). Although both single and multiple exposures to either extract increase BALF eosinophils and neutrophils compared to controls, multiple extract exposures significantly increased eosinophils but not neutrophils compared to a single exposure.

Airway hyperresponsiveness

Non-specific airway hyper-reactivity and bronchoconstriction are frequent symptoms of asthma. The cholinergic agonist methacholine (Mch) has been widely used in studying non-specific bronchial reactivity. One day after single or multiple exposures to 20 µg of extract, mice were challenged with increasing doses of Mch (6.25, 12.5, and 25 mg/ml). Multiple exposures to PCE induced a significant increase in airway hyper-reactivity compared to mice receiving a single PCE exposure (Figure 4) suggesting an adaptive immune response. Although only multiple exposures to HDM were statistically significant compared to the control, airway responses to Mch challenge were similar regardless of the number of HDM exposures.

Serum total IgE

Elevations in serum total IgE indicate an atopic status and are frequently associated with allergic airway disease. Serum total IgE levels were dose-dependently elevated in HDM- and PCE-treated mice throughout the dose range (Figure 5A). The lowest dose that induced a significant increase in total IgE compared to 0 µg dose controls was 20 µg of HDM and 10 µg of PCE. PCE induced significantly higher levels of serum total IgE compared to HDM at the 10, 40, and 80 µg doses.

Serum antigen-specific IgE

As an indirect measure of functional antigen-specific IgE, the release of the preformed mediator β -hexosaminidase was measured using the RBL cell assay. The extract dose resulting in 10% of total β -hexosaminidase release was calculated to provide a basis for com-

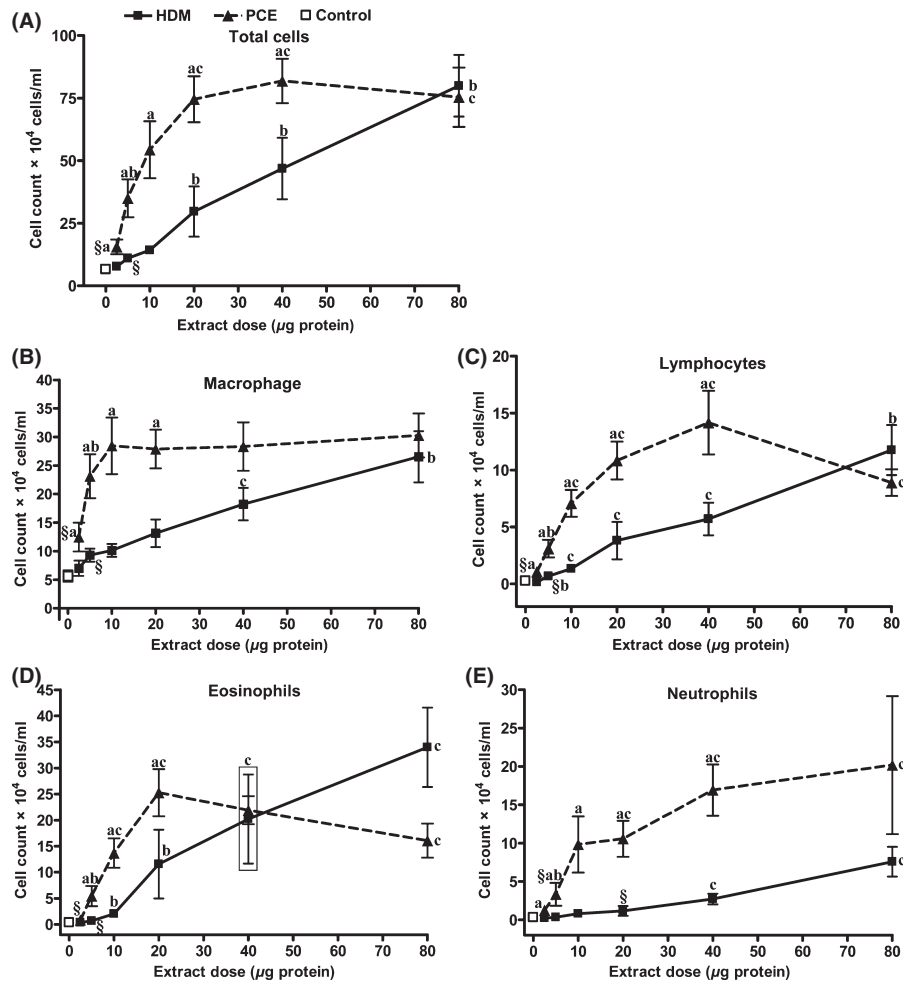


Fig. 3 Total cell counts (A) and differential cell counts (Macrophage (B), Lymphocytes (C), Eosinophils (D), and Neutrophils (E)) in bronchoalveolar lavage fluid of house dust mite (HDM)- or PCE-exposed mice collected 3 days after the final exposure. Symbols indicate (§) the lowest significant dose compared to Hanks' balanced salt solution (0 dose control), statistically significant differences (a) between extracts at the same dose, (b) from all lower doses within treatment, (c) from 2.5-μg and 5-μg doses within treatment. ($P < 0.05$). Error bars represent standard error of the mean. Control $n = 12$; HDM and PCE $n = 5-6$

paring the extracts' ability to induce antigen-specific IgE (Figure 5B). The calculated dose of PCE that would result in the development of sufficient antigen-specific IgE levels to induce 10% of total mediator release was 5.11 μg. This was lower than that required for HDM (13.17 μg) to achieve 10% of total mediator release. Additionally, PCE induced a significantly higher % of total mediator release compared to HDM at the 5, 10, 40, and 80 μg dose levels. The data indicate that exposure to PCE may lead to an increased risk of developing an antigen-specific IgE response which could result in increased mast cell degranulation (mediator release).

When the extracts were assayed for endogenous chitinase activity (Figure 5C), HDM demonstrated little or no enzymatic activity compared to the control (no extract added). However, PCE demonstrated elevated chitinase activity compared to both HDM and the control.

Protease activity

Fungal extract protease activity can amplify the inflammatory responses of allergic disease through protease-activated receptors on epithelial cells (Reed and Kita, 2004). The extracts were assayed for proteolytic activity to investigate how differences might impact extract-induced allergic responses. The proteolytic activity in PCE was higher than that of HDM but both demonstrated lower protease activity than the trypsin control with activity ranging from 38 to 71% and 25 to 55% of trypsin proteolytic activity, respectively (Figure 6).

Discussion

Previous studies in our laboratory (Chung et al., 2005) and the study presented here have found that *P. chrysogenum* extract has the capacity to induce

Table 1 Comparison of total and inflammatory cell counts following 1 or 3 IA exposures: bronchoalveolar lavage fluid (BALF) neutrophil and eosinophil cell counts 2 days after the final IA exposure to 20 μ g of extract protein. Control animals received 3 IA exposures to Hanks' balanced salt solution (HBSS)

BALF cells	Treatment			Number of exposures
	Control (0 μ g) ^a	HDM	PCE	
Total cells	3.95 (0.19)	32.10 (2.30)*†	11.97 (2.72)	1
($\times 10^4$ cells/ml)		48.17 (7.86)*‡	41.67 (6.71)*‡	3
Neutrophils	0.19 (0.10)	19.24 (2.63)*†	6.41 (2.18)	1
($\times 10^4$ cells/ml)		20.28 (5.09)*†	8.25 (1.69)	3
Eosinophils	0.01 (0.01)	0.83 (0.28)*	0.28 (0.13)*	1
($\times 10^4$ cells/ml)		9.51 (2.61)*‡	14.06 (3.72)*‡	3

Data shown are the average (standard error of the mean).

HDM, house dust mite.

^aControl animals were exposed to HBSS 3 times.

*Significant difference compared to control group.

†Significant difference compared to other treatment. $P < 0.05$.

‡Significant difference compared to single exposure within treatment.

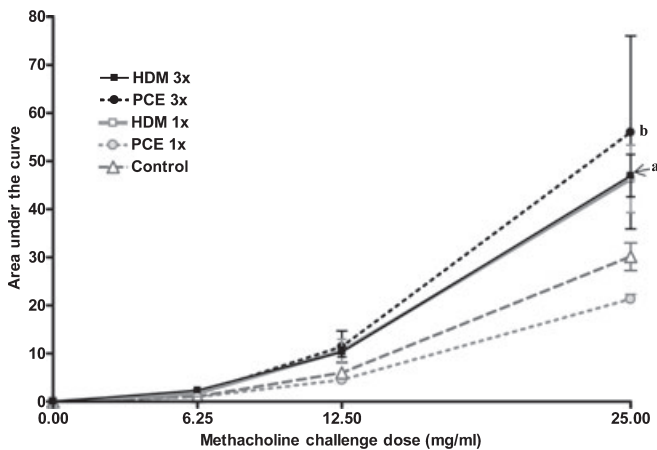


Fig. 4 Methacholine challenge. Mice exposed to either a single (1 $\times$) or multiple (3) doses of PCE or house dust mite (20 μ g extract protein/exposure) were challenged with nebulized methacholine 24 h after the last exposure. Data are presented as area under the curve $\pm$ standard error. $n = 4-6$. Symbols indicate statistically significant differences (a) different from control, (b) different from single exposure/same treatment ($P < 0.05$)

dose-dependent allergic and asthmatic responses in a mouse model. In the present study, the relative allergenicity of the *P. chrysogenum* extract was compared to HDM-induced responses based on various endpoints with an emphasis on those that are characteristic of human allergic responses, e.g. serum total and antigen-specific IgE and inflammatory cell (neutrophils and eosinophils) influx into the lungs (summarized in Table 2). In general, PCE induced the most robust response at the lowest extract dose, including some allergen-specific responses (serum total IgE, antigen-specific IgE and BALF eosinophils). If only the lower doses (2.5–10 μ g) are taken into account, which generally appears to be the linear portion of the

response, PCE is clearly the most potent on a microgram of protein basis. This is consistent with the lowest significant dose and the response magnitude. In the functional antigen-specific IgE assay, the same response level required 2/5th as much PCE compared to HDM.

There are a number of factors that might affect the differential responses seen between the two extracts studied. One such factor might be differences in the quality or quantity of the 'allergens' among the extracts. As was shown in murine model of allergic asthma, *Aspergillus fumigatus* allergens differ in their ability to induce IgE, eosinophils, and airway hyper-responsiveness and can differentially affect the allergic asthma pathogenesis (Kurup et al., 2001). Furthermore, the authors found that in addition to the allergens, other extract components such as enzymes and toxins contributed to the overall responses leading to allergic asthma.

Protease activity has been shown to play a role in allergic responsiveness (Kheradmand et al., 2002) possibly by causing epithelial cell damage (Kauffman, 2003) or by the amplification of allergic inflammatory responses through protease-activated receptors on epithelial cells (Reed and Kita, 2004). Although the protease activity in PCE was slightly higher than in HDM, the protease activity in both extracts was substantially less than the trypsin control. Therefore, it does not appear to be a major factor in this study.

Chitinases are widely distributed in living organisms and are thought to primarily play a role in pathogen defense. Recently, elevated levels of acid mammalian chitinase were observed in the lung tissue of asthmatic patients (Zhu et al., 2004) and exogenous chitinase (*Streptomyces griseus*) was shown to activate Protease-activated Receptor-2 in human airway epithelial cells (Hong et al., 2008). Although chitinase activity in PCE was elevated compared to HDM, the protease (trypsin) control and the background control, it is not clear from our current study what impact this may have had on allergy development. This area merits further examination.

A single exposure to either HDM or PCE (20 μ g) resulted in a significant increase in BALF neutrophils (Table 1) but to varying degrees. The ability of these allergenic extracts to induce a prominent neutrophilic inflammatory response in naïve individuals could enhance their potential to induce and/or exacerbate allergic asthma (Fahy et al., 1995; Jatakanon et al., 1999). Although in the single-dose study multiple exposures (3 $\times$) did not change the neutrophil influx nor did 4 $\times$ exposures to 20 μ g of PCE (dose-response study), HDM-induced neutrophil counts were higher following three exposures than four exposures. Our data do not allow us to distinguish between possibility of cohort-to-cohort variability and an adaptive response resulting from the additional exposure.

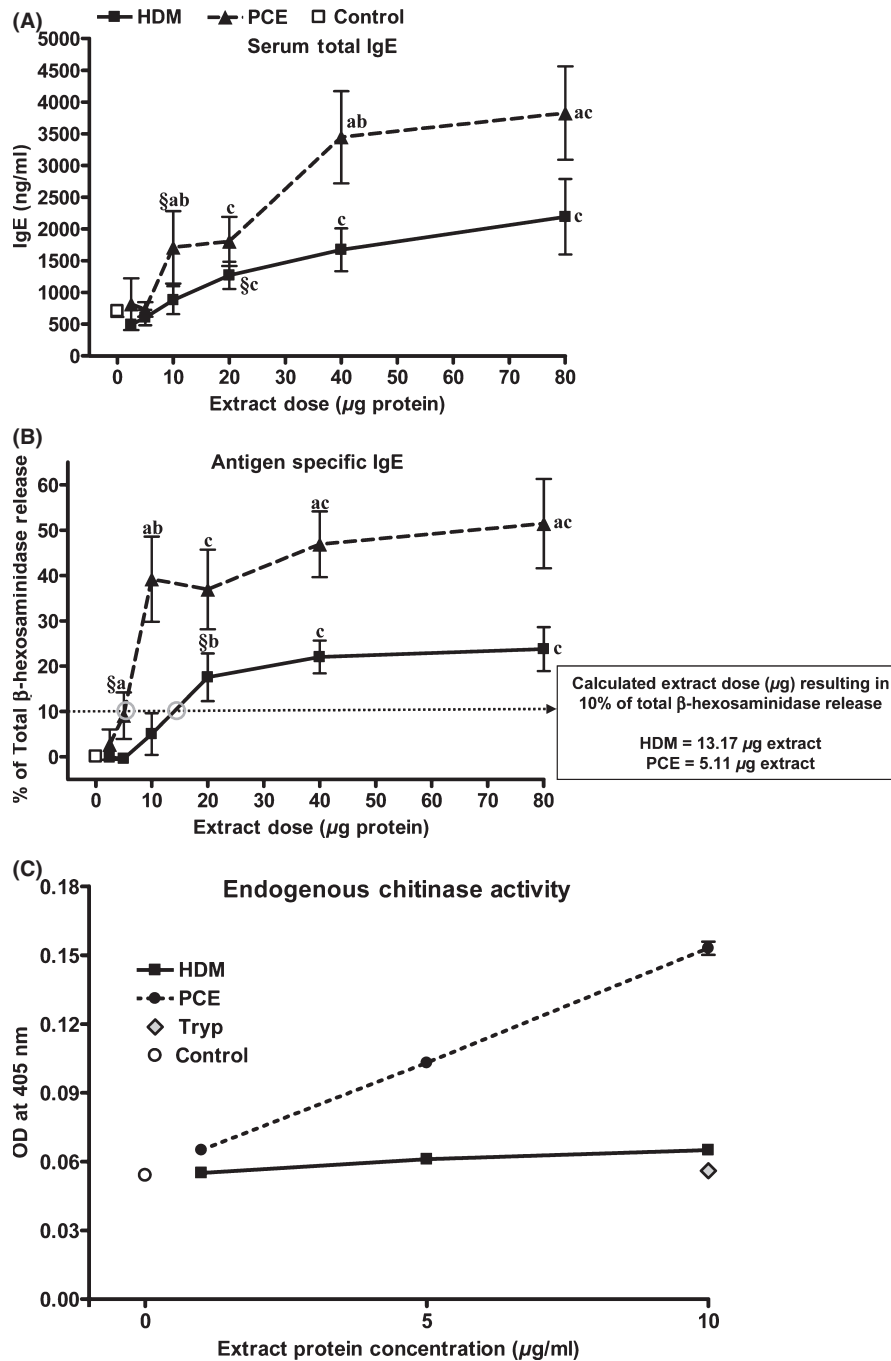


Fig. 5 (A) Serum total IgE levels of house dust mite (HDM)- or PCE-exposed mice collected 3 days after the final exposure. (B) Dose-response for percent of total β -hexosaminidase release from rat basophilic leukemia cells (index of antigen-specific IgE) after incubation with serum (1:4 dilution) obtained from HDM- or PCE-exposed mice 3 days after the final exposure. The extract doses calculated to result in 10% of total β -hexosaminidase release (dashed line) are indicated by circles on the dose-response curve. (C) The endogenous chitinase activity in the PCE and HDM extracts. Symbols indicate (§) the lowest significant dose compared to Hanks' balanced salt solution (0 dose control), statistically significant differences (a) between extracts at the same dose, (b) from all lower doses within treatment. (c) from 2.5- μg and 5- μg doses within treatment. ($P < 0.05$). Error bars represent standard error of the mean. Control $n = 12$; HDM and PCE $n = 5-6$

Co-exposure to endotoxin is another factor that may influence allergic responsiveness. In human studies, neither pre-allergen challenge exposure to low levels of endotoxin (lipopolysaccharide (LPS), 2 μg) (Sohy et al., 2006) nor LPS challenge (100 μg cumulative dose) of healthy and asthmatic individuals (Kitz et al.,

2006) was found to have a significant effect. However, in an ovalbumin allergy/asthma mouse model, co-exposure to 100 ng LPS during sensitization increased allergic responses (Kim et al., 2007). Although Dela-ayre-Orthez et al. (2004) found no significant effect with 10 ng LPS during sensitization in an ovalbumin

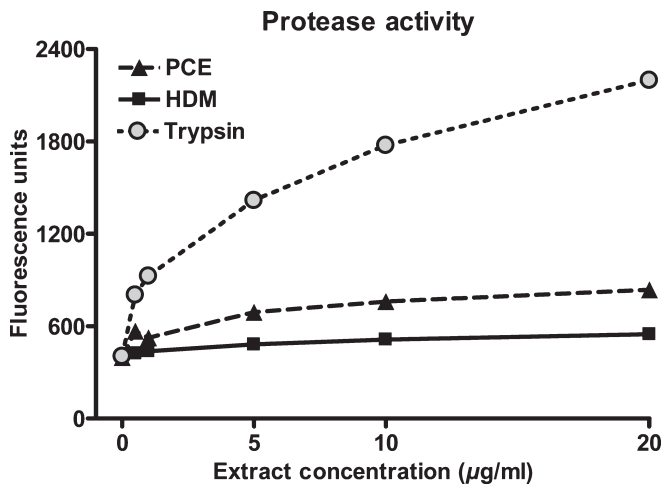


Fig. 6 Extract protease activity compared to trypsin protease activity control

allergy/asthma mouse model, administration of 1 ng LPS during challenge did increase eosinophils, total cells, and IL-5. In our study, the HDM extract had the highest level of endotoxin (0.27 EU in 80 µg of extract protein) that equates to ~ 0.027–0.054 ng LPS. This is well below the levels expected to have an impact on allergic responses. Furthermore, PCE (0.01–0.05 ng LPS) generally induced significant increases in BALF eosinophils and neutrophils and antigen-specific IgE at lower doses than HDM. This suggests that the endotoxin levels in these extracts had little if any effect on the response outcome in this study.

Exposure assessment and threshold-sensitizing dose are critical in understanding the development of allergic disease with regard to a given agent. Studies have found that an exposure to ≥ 2 µg *Der p* I/g dust is a risk factor for HDM sensitization in atopic children

(Lau et al., 1989; Platts-Mills et al., 1992). Additionally, Arbes et al. (2003) estimated that most beds in US homes have detectable levels of HDM with 2 µg HDM allergen/g dust in $46.2 \pm 2\%$ of beds and 10 µg/g dust in $24.2 \pm 2.1\%$ of beds. Even though dust allergen load and aspiration exposures (as in the current study) are not equivalent, the fact that there were 1.96 µg of Der I allergens in 10 µg HDM extract protein suggests that HDM dosing in our study was within the range of human exposure.

The sensitizing exposure threshold dose like the one defined for HDM is not available for most allergens including molds. Generally, *Penicillium* and *Aspergillus* species have been grouped for the purpose of exposure assessment further complicating the determination of a sensitizing exposure dose. More recently, home assessment studies have identified (speciated) and quantified various molds in house dust using MSQPCR (Haugland et al., 2002) including *P. chrysogenum* (Vesper et al., 2006, 2008). This data plus dose-response comparisons like the one presented in this study may provide insight into sensitizing dose thresholds.

Our data suggest that *P. chrysogenum* is a robust allergen source and may be a more potent allergen source than HDM in this mouse model. However, it must be noted that the allergen load in our *P. chrysogenum* extract is unknown. Furthermore, the HDM extract used in this study is lyophilized whole bodies not including fecal matter that has a higher concentration of allergens. Therefore, the allergen load in our HDM extract might be lower than that would be seen in a typical human exposure. The evaluation of an agent's allergenic potential must include sensitization thresholds and exposure levels that are critical factors in allergic disease development. At this time, these factors are unknown for most allergens including

Table 2 Summary of endpoints characteristic of human allergy based on the lowest dose that induced a significant response compared to the 0-µg dose control ($P < 0.05$) and the magnitude of the response^a at that dose

Source	Endpoint	Relative potency		
		Lowest dose ^b for significant response (response magnitude)		
		HDM		PCE
BALF	Total protein	10 (81.61 ± 4.38)	<	2.5 (92.56 ± 3.76)
	Lactate dehydrogenase activity	20 (32.84 ± 4.41)	=	20 (34.25 ± 4.99)
	Total cells ($\times 10^4$ cells/ml)	5 (11.00 ± 1.43)	<	2.5 (15.56 ± 2.94)
	Macrophage count ($\times 10^4$ cells/ml)	5 (9.30 ± 1.15)	<	2.5 (12.45 ± 2.52)
	Lymphocyte count ($\times 10^4$ cells/ml)	5 (0.68 ± 0.23)	<	2.5 (1.05 ± 0.39)
	Neutrophil count ($\times 10^4$ cells/ml)	20 (1.17 ± 0.64)	<	5 (3.33 ± 1.50)
	Eosinophil count ($\times 10^4$ cells/ml)	5 (0.67 ± 0.17)	<	2.5 (0.82 ± 0.23)
	Total IgE (ng/ml)	20 (1267.90 ± 214.64)	<	10 (1708.65 ± 571.21)
Serum	Antigen-specific release (% total mediator release)	20 (17.55 ± 5.28)	<	5 (9.07 ± 5.13)
	Antigen-specific IgE ^c (µg extract)	13.17	<	5.11

HDM, house dust mite; BALF, bronchoalveolar lavage fluid.

^aQuantified response at lowest significant dose (µg extract) compared to control (0 µg).

^bLowest dose in µg extract protein.

^cCalculated dose that would induce sufficient antigen specific IgE resulting in 10% of total mediator release in rat basophilic leukemia assay.

molds/fungi. Importantly, the data presented in the current study suggest a threshold dose for the induction of allergic responsiveness to *P. chrysogenum*. Additionally, *P. chrysogenum* as well as other molds may play an important role in allergic asthma development in our society.

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