

RESEARCH ARTICLE

Assessing the allergenic potential of molds found in water-damaged homes in a mouse model

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Abstract

Damp/moldy indoor environments, which have resulted from flooding events and may increase as a result of climate change, have been associated with asthma exacerbation. Certain molds found in significantly higher or lower concentrations in asthmatics' homes compared to control homes have been categorized as Group 1 (G1) and Group 2 (G2) molds, respectively. We have compared the allergic potential of selected G1/G2 molds to house dust mite (HDM) in a mouse model. BALB/c mice were exposed to mold (0–80 µg) or HDM (20 µg) extract by intratracheal aspiration either 4X over 4 weeks (allergenicity) or 1X (non-specific responses). Airflow limitation (methacholine challenge) was measured (Day 1) and serum and bronchoalveolar lavage fluid were collected (Day 2) after the final exposure. The G1 molds induced low-to-moderate responses and required higher doses to achieve antigen-specific IgE results similar to those induced by HDM. Compared to HDM responses, the G2 mold in this study required lower doses to induce a similar response. Acute exposure responses suggest some molds may exacerbate asthmatic responses. These studies demonstrate the differing capacities of molds to induce responses associated with allergic asthma, including differences in the threshold dose for allergy induction. Therefore, molds must be evaluated individually for allergic/asthmatic potential. These studies along with our previous studies with G1 (*Stachybotrys chartarum*)/G2 (*Penicillium chrysogenum*) molds suggest that the G1/G2 categorization is not indicative of allergic potential but they do not preclude this categorization's utility in determining unhealthy building dampness.

Keywords

Allergenicity, allergic asthma, house dust mite, mouse model, relative allergenicity

History

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Introduction

Mold-related health effects have gained national attention due to hurricanes and flooding in the USA along the Gulf coast and the eastern seaboard from hurricanes as well as inland from flooding events. Additionally, climate change is projected to increase the prevalence and exposure to molds, pollen, and other agents due to increased atmospheric CO₂ concentrations and humidity in some areas. Furthermore, it is anticipated that climate change will result in increased dampness, water intrusion and fungal growth in the indoor environment. Damp/moldy environments have been associated with asthma exacerbation.

Molds are ubiquitous in the environment and exposures contribute to various human diseases including allergic lung diseases. The Institute of Medicine (IOM) reports [National Academy of Sciences (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 & Fisk (2007) estimated that 21% of current asthma in USA may be attributed to dampness and molds. 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 epidemiologic evidence of an association between fungal exposure and asthma severity.

In studies in the Cleveland area, certain molds were found to be statistically more common in water-damaged homes (Group 1 molds: G1), and other molds were found in every home (Group 2 molds: G2) (Vesper et al., 2004). In a follow-up study, these mold groupings were used to evaluate mold populations in asthmatics' water-damaged homes. Mold populations were measured in dust samples by mold-specific quantitative polymerase chain reaction (MSQPCR) and traditional culturing methods (Vesper et al., 2006).

Of the 33 molds examined, five G1 molds were found in significantly higher concentrations in asthmatics homes compared to control homes (without water damage or asthma). Additionally, seven G2 molds were found in significantly lower concentrations in asthmatics' homes compared to control homes (Vesper et al., 2006).

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Subsequently, these water-damaged homes were divided into remediated and non-remediated asthmatic's homes and the asthmatic condition of the children was monitored (Kercsmar et al., 2006). The moderately severe asthmatic children had a significant decrease in symptom score and symptom days following remediation of their water-damaged homes (Kercsmar et al., 2006). During the period from 6-months post-randomization to the end of the study, which was primarily post-remediation, asthmatics receiving home interventions had a lower rate of asthma exacerbations compared to control asthmatics (1/29 versus 11/33, respectively; $p=0.003$). Populations of some specific molds (G1 molds) were directly associated with water-damaged homes of asthmatic children and removal of these molds resulted in a significant improvement in asthma symptoms. Thus, it appeared that G1 molds, but not G2 molds, may be associated with asthma, at least in these Cleveland water-damaged homes. Remediation of water-damage would affect many things in the home besides mold concentrations. Therefore, a mold allergy animal model was used to elucidate the association between specific molds and asthma and provide information on the relative potency of different molds.

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 be seen after challenge characterized by mucus hypersecretion, bronchoconstriction, increased airway resistance, and eosinophilic airway inflammation. Additionally, individuals with allergic lung disease and asthma may exhibit airway hyperresponsiveness (AHR) to non-specific stimuli, e.g. histamine or methacholine (Mch). Some late-phase and chronic components are cell-mediated and categorized as type IV hypersensitivity responses.

We hypothesized that the association between damp homes and allergic lung disease or asthma is related to the increased potency of allergens in molds prevalent in that environment. Therefore, the goal of the studies described here were (i) to elucidate the association between specific molds and allergy/asthma using a mouse model and (ii) to assess the relative allergenicity of these molds by comparing responses to those induced by the well-characterized, indoor allergen house dust mite (HDM).

Materials and methods

Animals

Fifty day-old female BALB/c mice (Charles River Laboratories, Raleigh, NC) were group-housed in polycarbonate cages with hardwood chip bedding in an environmentally controlled, Association for Assessment and Accreditation of Laboratory Animal Care International accredited vivarium. Mice were maintained on a 12-h light/dark cycle and allowed access to food (Purina Rodent Lab Chow, St. Louis, MO) 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 US Environmental Protection Agency's National Health and Environmental Effects Laboratory's Institutional Animal Care and Use Committee.

Fungal antigen preparation

Three G1 mold extracts [*Scopulariopsis brevicaulis* (SBE), *Trichoderma viride* (TVE) and *Wallemia sebi* (WSE)] and one G2 mold extract [*Cladosporium cladosporioides* types 1 and 2 (CCE)] used in these studies are listed in Table 1. The fungi were grown on nylon filters (0.45 μ m pore size; GE Osmonics, Inc., Minnetonka, MN) placed on potato dextrose agar plates and/or tryptic soy agar plates (Difco Media) and incubated for 2–3 weeks. The fungal mass (mycelium and spores) of each isolate was extracted by grinding with a sterile mortar and pestle for ~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) + 0.05% (v/v) Tween-80 (Thermo Fisher Scientific, Pittsburgh, PA). 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) using YM-3 membranes (molecular weight >3000 Da cutoff) and filter sterilized with a 0.2- μ m syringe filter. Each individual crude antigen extract was assayed for total protein concentration using a Pierce BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL) with bovine serum albumin as a standard according to manufacturer's procedures. The extracts were stored at –80 °C until use. The endotoxin level at the highest dose (80 μ g extract protein) of each extract was measured using a Limulus amoebocyte lysate test kit (BioWhittaker, Inc., Walkersville, MD) (Table 1).

Lyophilized whole-body extracts of HDM (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) purchased from Greer Laboratories (Lenoir, NC), 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 extract protein) was 0.152 EU (0.02–0.03 ng).

Experimental design

The assessment of allergenic potential for the selected G1 and G2 molds was carried out in two studies each of which included HDM (four exposures of 20 μ g extract protein) as a well-characterized indoor allergen control. Study I

Table 1. Extracts.

Fungi	Exposure protocol ^a		Category ^b	Source	Endotoxin level ^c (in 80 µg extract dose)	
	4X	1X				
<i>C. cladosporioides</i> types 1 and 2	CCE	HB/CC	Group 2	ATCC #6721	0.046 EU	0–0.01 ng
<i>S. brevicaulis</i>	SBE	HB/SB	Group 1	UAMH #7771	0.089 EU	0.01–0.02 ng
<i>T. viride</i>	TVE	HB/TV	Group 1	UAMH #6280	0.138 EU	0.01–0.03 ng
<i>W. sebi</i>	WSE	HB/WS	Group 1	UAMH #7897	0.061 EU	0–0.01 ng
HDM						
<i>D. farinae</i> and <i>D. pteronyssinus</i>	HDM	–	–	Greer Laboratories, Lenoir, NC	0.152 EU	0.02–0.03 ng

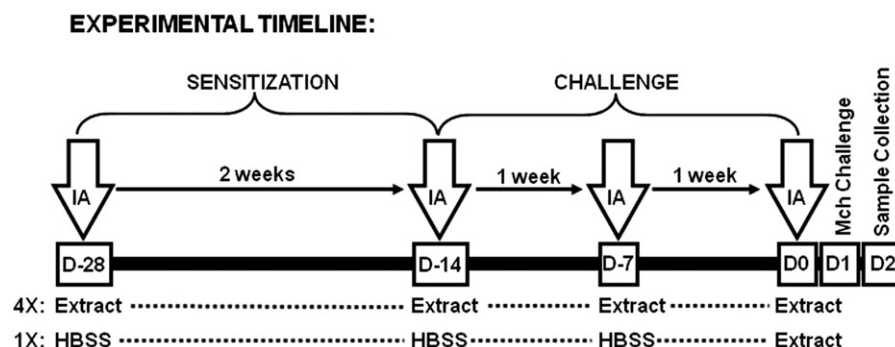
^aAbbreviations used as mold extract identifiers for multiple (4X) and single (1X) exposures.

^bCategorization by Vesper et al. (2006).

^cEndotoxin was assessed at the highest dose level (80 µg extract protein).

UAMCH = University of Alberta Microfungus Collection and Herbarium.

Figure 1. Experimental timeline. The multiple exposure protocol included four exposures to the extracts over a 4-week period. For the single exposure protocol, the first three exposures were to HBSS and the fourth exposure to extract. The 0-µg dose control animals were instilled with HBSS only.



included: CCE and SBE plus HDM. Study II included: TVE and WSE plus HDM. Groups of five female BALB/c mice were exposed by intratracheal aspiration (IA) to 5, 10, 20, 40, or 80 µg of mold extract protein in 50-µl HBSS four times over a 4-week period (4×) to induce and assess allergic sensitization, as previously described (Ward et al., 2000). Multiple exposure mice are identified by the abbreviation for the dosing agent (CCE, SBE, TVE, and WSE). To distinguish allergy induction (multiple exposures) from non-specific inflammatory responses, groups of four mice were exposed to HBSS for three times with the final exposure to the fungal extract (1×). These mice 1× (single exposure) are identified as HB/CC, HB/SB, HB/TV, and HB/WS indicating initial dosing with HBSS and a final dose of the fungal extract. Additionally, 15–16 mice were instilled with 50 µl of HBSS only (0 µg dose control) four times. Figure 1 shows the experimental timeline. Briefly, mice were anesthetized by inhalation of a mixture of 3% isoflurane and 97% oxygen. Antigen extract was deposited into the oropharynx, after which mice aspirated the extract when their noses were gently occluded with a fingertip. Airway responses to Mch challenge were assessed 1 day after the final exposure. Serum and bronchoalveolar lavage fluid (BALF) were collected 2 days following the final exposure. Lavage fluid was assessed for total cell and differential cell counts. Serum was assessed for total and extract-specific IgE. The responses of allergen extract-treated mice were compared to results obtained from 0 µg dose control animals, among doses within treatments, and among treatments within a dose.

Airway responsiveness to Mch aerosol

As previously described by Cho et al. (2009), airway responsiveness to Mch aerosol was measured in unrestrained

mice using whole-body plethysmography (Buxco Electronics, Inc., Wilmington, NC) expressed as enhanced pause (Penh) 1 day after the final IA exposure. Mch responsiveness was analyzed across treatment groups and within individual mice using SAS software (SAS Institute Inc., Cary, NC). The data are presented as area under the curve (AUC).

Blood collection and bronchoalveolar lavage

Blood and BALF samples were collected on Day 2 after the final exposure. Briefly, mice were anesthetized with sodium pentobarbital 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, and the serum was stored at –80 °C until analysis. The lungs were lavaged twice with 1-ml aliquots of HBSS, and the pooled BALF aliquots were stored on ice. The BALF was centrifuged at 100 g for 15 min at 4 °C and 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). Additionally, 100–150 µl of resuspended cells were adhered onto glass slides at 200 rpm for 10 min using a Cytospin II centrifuge (Shandon Inc., Pittsburgh, PA). The BALF cells on glass slides were stained with Wright-Giemsa (Thermo Fisher Scientific, Pittsburgh, PA) on a Hema-Tek 2000 slide stainer (Miles Laboratories, Inc., Elkhart, IN), and were differentially counted at 200 cells per slide (one slide per animal).

Total IgE enzyme-linked immunosorbent assay

Total IgE enzyme-linked immunosorbent assay (ELISA) was performed as previously described by Chung et al. (2005). Purified monoclonal anti-mouse IgE (BD Biosciences/

PharMingen, San Diego, CA) was used as the capture antibody and biotinylated rat anti-mouse IgE (BD Biosciences/PharMingen, San Diego, CA) as the detection antibody. Optical density was read on a SpectraMax 340 PC Plate Reader (Molecular Devices Corp., Menlo Park, CA) at a wavelength of 650 nm. SoftMax Pro[®] version 2.6.1 (Molecular Devices Corp., Menlo Park, CA) 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 [American Type Culture Collection (ATCC), Rockville, MD] and incubated for 18 h at 37°C in a 5% CO₂ 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 μ g) and incubated for 1 h at 37°C in a 5% CO₂ humidified incubator. To determine total β -hexosaminidase (mediator) release cells were lysed by incubating them with 1% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO) in Tyrode's buffer. To determine background or spontaneous mediator release, cells were incubated with buffer alone. The mediator measured, β -hexosaminidase, is a chitinase. Because some fungi also produce chitinases an additional control was used to identify extract-specific (endogenous) chitinase activity; CCE, SBE, TVE, WSE, and HDM extracts were added to cells that were not passively sensitized (no serum). 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., Menlo Park, CA) at a wavelength of 405 nm and data collected by SoftMax Pro[®] software version 2.6.1 (Molecular Devices Corp., Menlo Park, CA). Data are presented as percent total release following subtraction of either spontaneous release or extract-specific activity whichever results in the highest optical density reading. The extract dose resulting in 10% of total release was calculated by interpolation.

% of Total β -hexosaminidase Release

$$= \frac{\text{Sample Release OD} - \left[\frac{\text{Spontaneous Release OD or Extract-Specific Release OD}}{\text{Total Release OD} - \text{Spontaneous Release OD}} \right]}{\text{Total Release OD} - \text{Spontaneous Release OD}} \times 100$$

Protease assay

Extracts were analyzed for protease activity using the EnzChek7Protease Assay Kit (red fluorescence) (Molecular Probes, Eugene, OR). Triplicates of five concentrations (0–20 μ 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 2 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 (SpectraMax, Gemini XS; Molecular Devices Corp., Menlo Park, CA) using a filter with excitation = 590 ± 10 nm, emission = 645 ± 20 nm. Data are reported as fluorescence units and percent of trypsin activity control.

Statistical analysis

Responses, other than airway response to Mch challenge, were analyzed using a one-way analysis of variance (ANOVA). The one independent variable, group, was formed by combining the exposure with the concentration. This was done, as opposed to using a two-way ANOVA with the two independent variables exposure and concentration, because of the inclusion of a single control group, thereby obviating the use of the two-way ANOVA. Pair-wise comparisons, among the groups, were performed as subtests of the overall ANOVA.

The airway response to Mch challenge analysis was initiated with a calculation of AUC of the graph with the abscissa being the Mch dose and the ordinate the airway response. Subsequently, a one-way ANOVA was performed on the AUC as the dependent variable and the exposure as the independent variable. Pair-wise comparisons, among the exposures, were performed as subtests of the overall ANOVA.

In both analyses, the type I error rate was set at 0.05 for individual statistical tests. For the pair-wise comparisons, no adjustment was performed for multiple testing. This may result in an inflation of the overall type I error rate; however, it will maintain a reasonable type II error rate.

Results

Airway responsiveness

Non-specific airway hyperreactivity and bronchoconstriction are frequent symptoms of asthma. The cholinergic agonist Mch has been widely used in studying non-specific bronchial reactivity. One day after the final extract exposure, mice were challenged with increasing doses of Mch aerosol (6.25, 12.5, and 25 mg/ml). Airway responsiveness (Figure 2) of HDM-exposed mice demonstrated a significant increase in airflow limitation compared to controls (0 μ g) at the 40 μ g dose level. However, the average response below the 40 μ g dose level was different between the two studies. Multiple exposures to CCE (10 μ g lowest significant dose level) a G2 mold and SBE (80 μ g) a G1 mold induced a significant increase in airflow limitation compared to controls (0 μ g)

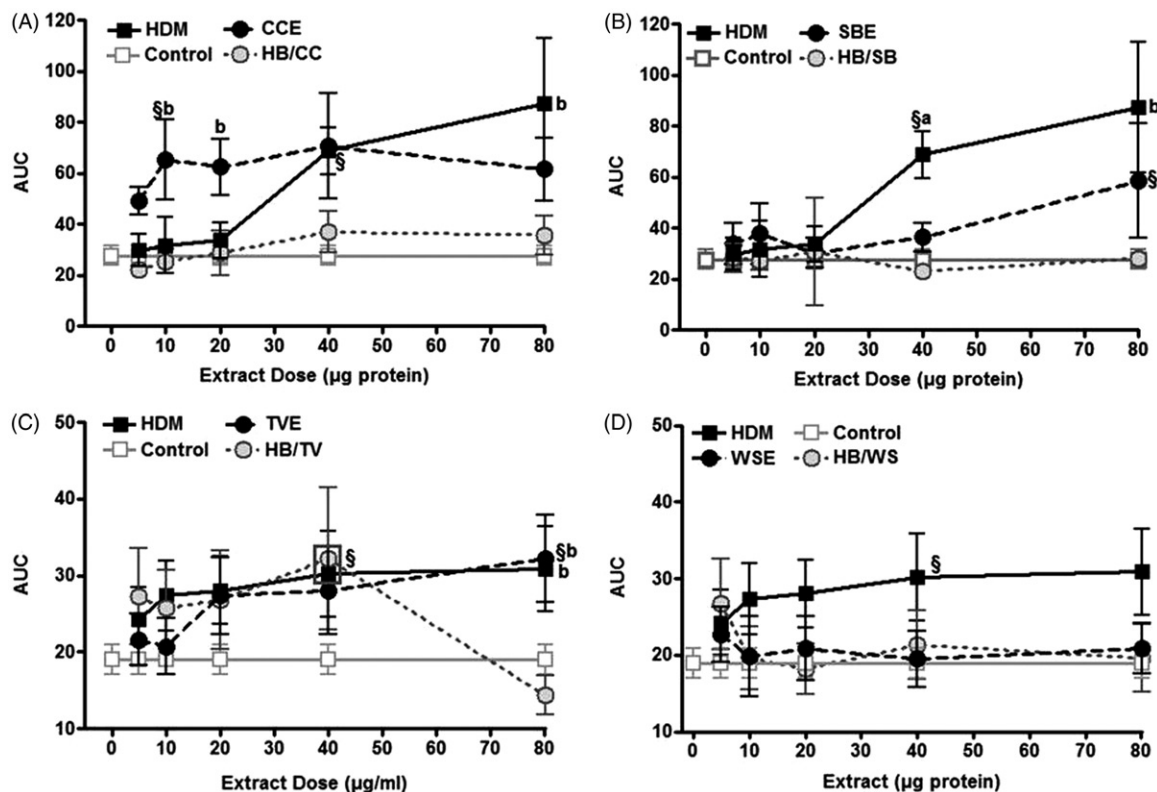


Figure 2. Airway response to Mch challenge presented as AUC following a single or multiple exposures to (A) *C. cladosporioides* extract (single exposure: HB/CC, multiple exposures: CCE), (B) *S. brevicaulis* extract (single exposure: HB/SB, multiple exposures: SBE), (C) *T. viride* extract (single exposure: HB/TV, multiple exposures: TVE), or (D) *W. sebi* extract (single exposure: HB/WS, multiple exposures: WSE). HDM = house dust mite extract. Symbols indicate (§) the lowest significant dose compared to control (0 µg dose), (a) indicates significant versus other treatments within dose and (b) indicates significant versus single exposure within dose ($p < 0.05$). Error bars represent standard error of the mean. Control, $n = 12$; multiple exposures, $n = 5-6$; single exposure, $n = 4$.

(Figure 2A and B). Although the response to CCE was significantly higher compared to the HDM-induced responses at in the lower dose range (5–20 µg) the CCE dose curve was relatively flat. Multiple exposures to TVE-induced elevated airway responses from 20 to 80 µg dose level similar to HDM, but were only significant compared to controls at the 80-µg dose level (Figure 2C). However, a single exposure to TVE (HB/TV) also resulted in similar responses to those of HDM (5–40 µg), suggesting that TVE may cause airway irritation. WSE airway responses from either single or multiple exposures were near control levels regardless of dose (Figure 2D).

BALF total and differential cell counts

On Day 2 (D2) BALF total and differential cell counts were assessed to determine the relative effect of the extracts to induce cellular influx into the lungs. Multiple exposures to CCE, SBE, TVE, and HDM extracts, but not WSE induced significant increases in BALF total cell counts compared to 0 µg dose controls ($p < 0.05$) (Figure 3). However, the dose required for the effect was lower and the quantity of cells higher for CCE and HDM (5 µg) extracts than SBE and TVE (20 µg). Additionally, a single exposure to either CCE (HB/CC, 5 µg) or TVE (HB/TV, 80 µg) induced a significant increase in total cells.

The BALF neutrophil response to a single exposure of CCE (HB/CC, 5 µg) and TVE (HB/TV, 40 µg) was significant

(Figure 4A and C). However, HB/CC induced significant increases at all dose levels compared to the 0-µg dose. Although, multiple exposures to CCE (5 µg), SBE (40 µg; Figure 4B), and TVE (80 µg) induced a significant increase in BALF neutrophils compared to the 0-µg dose, only CCE induced a neutrophil response similar to that of HDM. A single exposure to CCE was significantly increased compared multiple CCE and HDM throughout most of the dose range (10–80 µg). No neutrophil increase was seen for WSE (Figure 4D).

Eosinophilic airway inflammation is an endpoint characteristic of allergic asthma responses. Multiple exposures were required to induce significant increases in BALF eosinophils compared to 0 µg dose controls. However, the dose required to induce significant increases varied [CCE and HDM extract (5 µg), SBE (10 µg), and TVE (20 µg)]. CCE-induced responses were significantly higher than those induced by HDM ($p < 0.05$) (Figure 5A). Additionally, the HDM-induced eosinophil response was significantly higher than that of SBE across the dose range (Figure 5B) ($p < 0.05$). TVE-induced BALF eosinophil responses were dose-dependent across the dose-response range (Figure 5C). WSE-induced responses were similar to control responses (Figure 5D). It should be noted that the eosinophil response to HDM was different in the two studies. The response demonstrated in Study I (CCE and SBE) is more consistent with earlier studies using HDM.

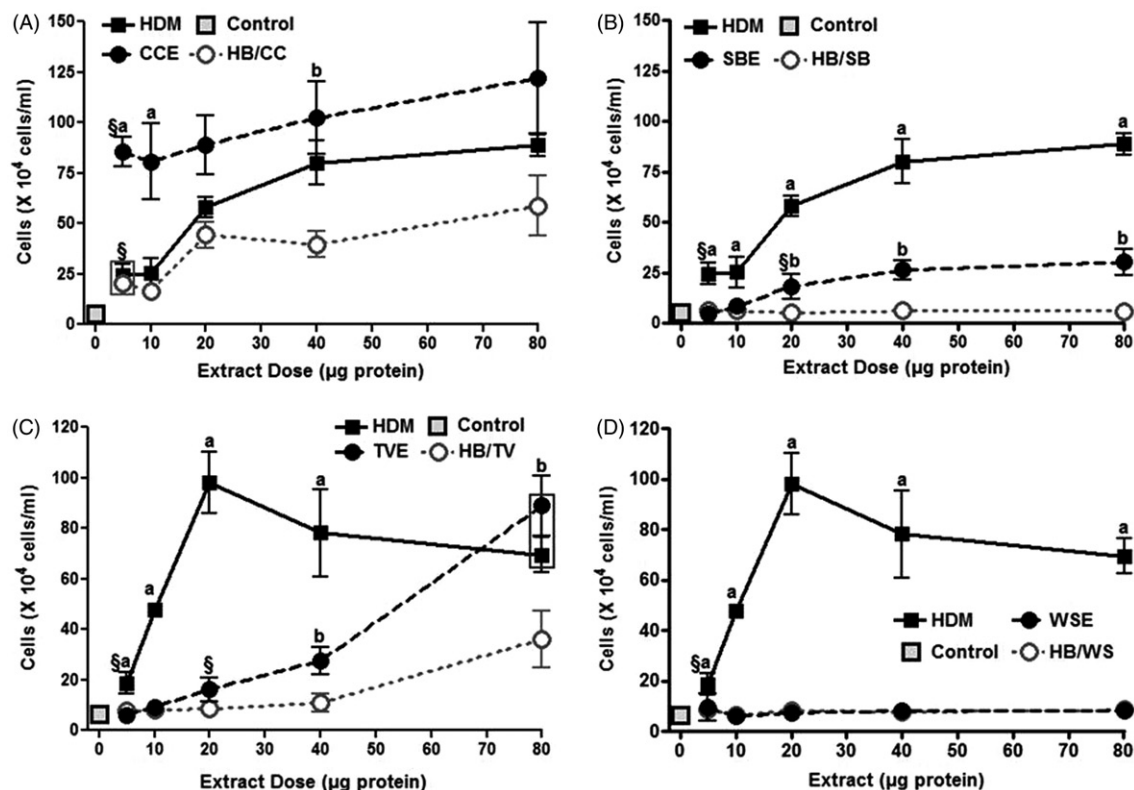


Figure 3. BALF total cell counts following a single or multiple exposures to (A) *C. cladosporioides* extract (single exposure: HB/CC, multiple exposures: CCE), (B) *S. brevicaulis* extract (single exposure: HB/SB, multiple exposures: SBE), (C) *T. viride* extract (single exposure: HB/TV, multiple exposures: TVE), or (D) *W. sebi* extract (single exposure: HB/WS, multiple exposures: WSE). HDM = house dust mite extract. Symbols indicate (§) the lowest significant dose compared to control (0 µg dose), (a) indicates significant versus other treatments within dose and (b) indicates significant versus single exposure within dose ($p < 0.05$). Error bars represent standard error of the mean. Control, $n = 12$; multiple exposures, $n = 5-6$; single exposure, $n = 4$.

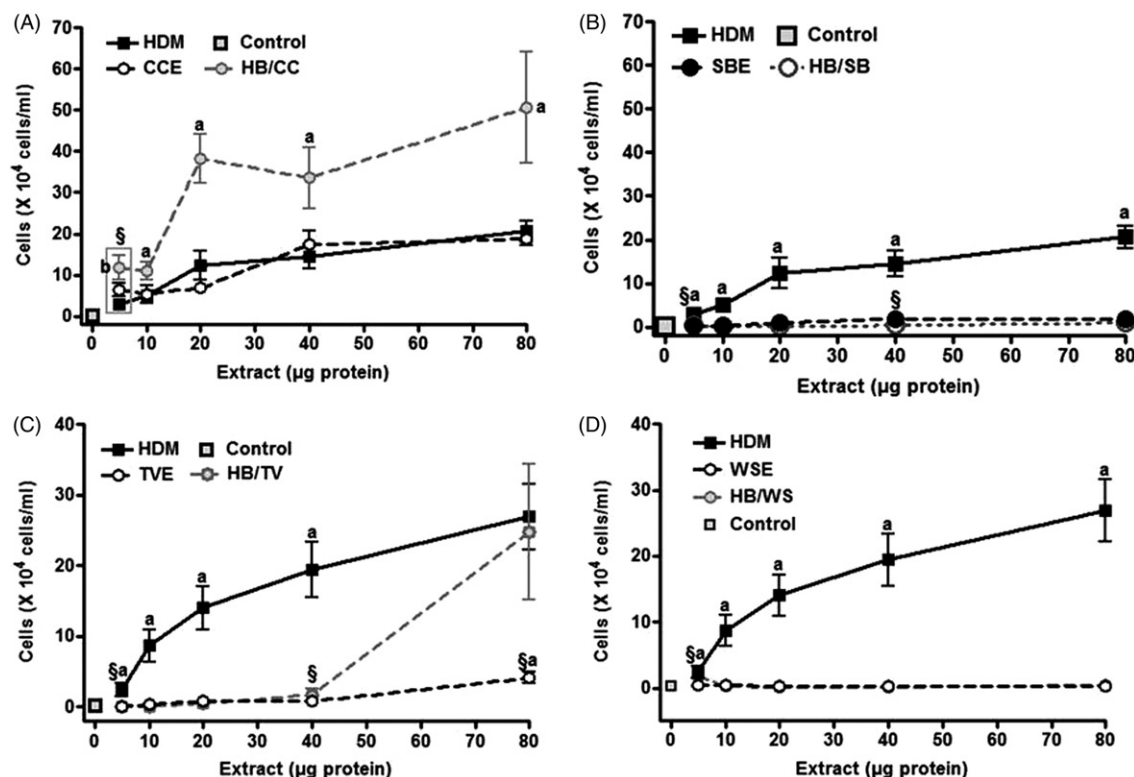


Figure 4. BALF neutrophil cell counts following a single or multiple exposures to (A) *C. cladosporioides* extract (single exposure: HB/CC, multiple exposures: CCE), (B) *S. brevicaulis* extract (single exposure: HB/SB, multiple exposures: SBE), (C) *T. viride* extract (single exposure: HB/TV, multiple exposures: TVE), or (D) *W. sebi* extract (single exposure: HB/WS, multiple exposures: WSE). HDM = house dust mite extract. Symbols indicate (§) the lowest significant dose compared to control (0 µg dose), (a) indicates significant versus other treatments within dose and (b) indicates significant versus single exposure within dose ($p < 0.05$). Error bars represent standard error of the mean. Control, $n = 12$; multiple exposures, $n = 5-6$; single exposure, $n = 4$.

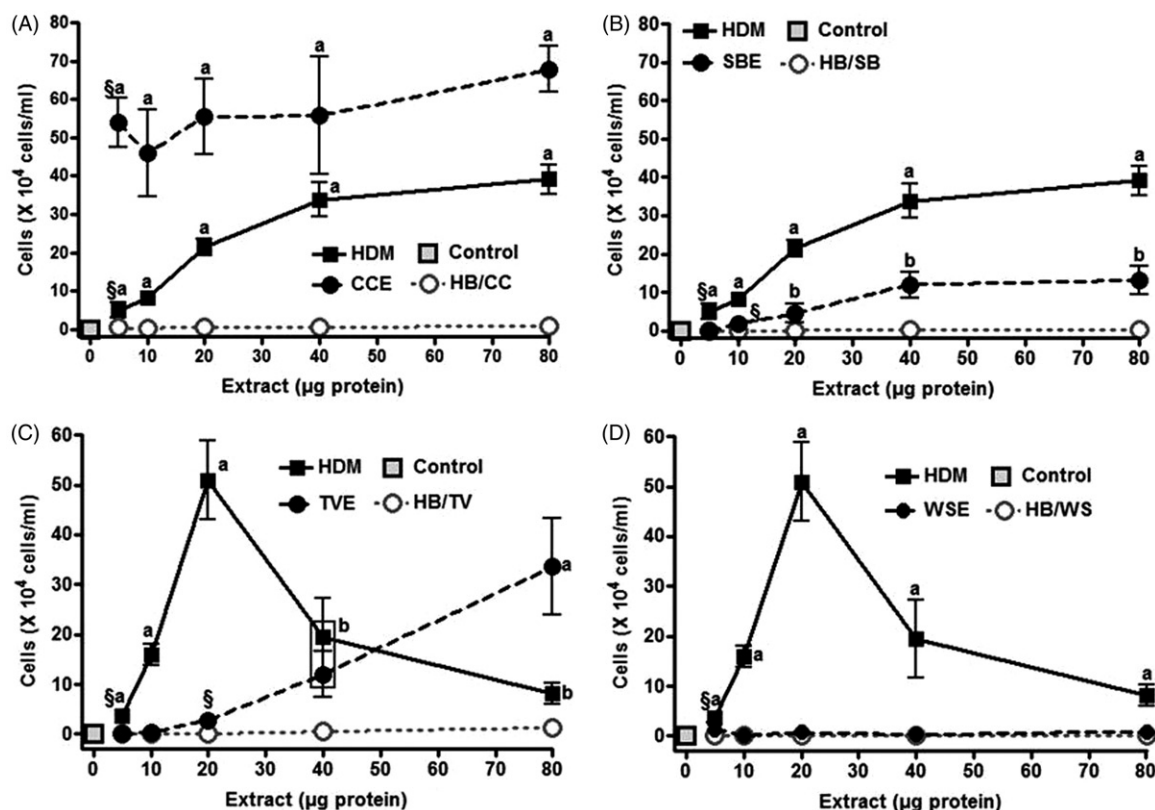


Figure 5. BALF eosinophil cell counts following a single or multiple exposures to (A) *C. cladosporioides* extract (single exposure: HB/CC, multiple exposures: CCE), (B) *S. brevicaulis* extract (single exposure: HB/SB, multiple exposures: SBE), (C) *T. viride* extract (single exposure: HB/TV, multiple exposures: TVE), or (D) *W. sebi* extract (single exposure: HB/WS, multiple exposures: WSE). HDM = house dust mite extract. Symbols indicate (§) the lowest significant dose compared to control (0 μ g dose), (a) indicates significant versus other treatments within dose and (b) indicates significant versus single exposure within dose ($p < 0.05$). Error bars represent standard error of the mean. Control, $n = 12$; multiple exposures, $n = 5$ – 6 ; single exposure, $n = 4$.

Serum total IgE

Elevations in serum total IgE indicate an atopic status, and are frequently associated with allergic airway disease. Multiple exposures to CCE (5 μ g), SBE (80 μ g), TVE (40 μ g), and HDM (20 μ g, Study I or 5 μ g, Study II) induced a significant increase in serum total IgE compared to the 0- μ g dose controls ($p < 0.05$) (Figure 6A–C). Additionally, CCE-induced responses that were significantly higher compared to HDM at 5, 10, and 80 μ g doses. Although the dose-response curve for SBE is similar to that induced by HDM exposures, the variability among the SBE-exposed mice resulted in no significant response except at the 80 μ g dose level. The TVE total IgE response increased significantly from the 40 μ g to the 80 μ g dose. The HDM response in the higher dose range in Study II was not consistent with previous studies in this laboratory. WSE did not induce significant levels of serum total IgE at any dose (Figure 6D). A single exposure did not induce significant increases in serum total IgE with the exception of TVE (HB/TV, 80 μ g) which induces a low level but significant increase in total IgE (Figure 6C).

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 rat basophil leukemia (RBL) cell assay.

Multiple exposures to the extracts with the exception of WSE induced significant increases in antigen-specific IgE compared to the 0- μ g dose controls.

The extract dose resulting in 10% of total β -hexosaminidase release was calculated to provide a basis for comparing the extracts' ability to induce antigen-specific IgE. The calculated dose for HDM was 19.0 μ g in Study I and the calculated doses for the fungal extracts were 4.1 μ g for CCE and 28.0 μ g for SBE (Figure 7A and B). In Study II, the calculated HDM dose was 6.5 μ g and the dose for TVE was 44.1 μ g (Figure 7C). WSE responses were similar to 0 dose controls (percent of total mediator release ranged from 0.1 to 1.0%) (Figure 7D). These calculated doses suggest that for two of the G1 molds it requires 1.5 $\times$ more SBE and 6.8 $\times$ more TVE to achieve the same response as HDM. However, only 0.2 $\times$ as much CCE (G2 mold) is required to achieve the same response as HDM.

Protease activity

Fungal extract protease activity can amplify the inflammatory responses of allergic disease through protease-activated receptors on epithelial cells (Reed & Kita, 2004). The extracts were assayed for proteolytic activity to determine if there are differences among the fungi and if there was an apparent impact on extract-induced allergic responses. The assay range was 0, 0.5, 1, 5, 10, and 20 μ g/ml of extract using trypsin as the control protease. The mold extracts

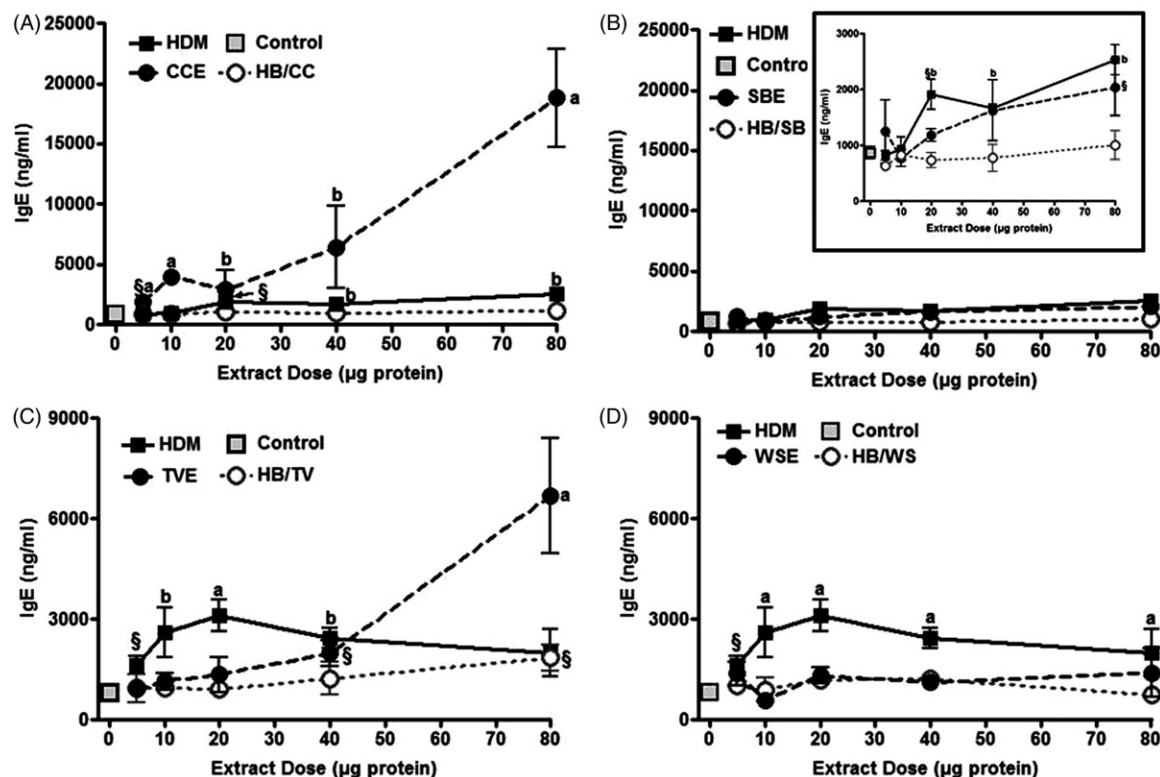


Figure 6. Serum total IgE was assayed for following a single or multiple exposures to (A) *C. cladosporioides* extract (single exposure: HB/CC, multiple exposures: CCE), (B) *S. brevicaulis* extract (single exposure: HB/SB, multiple exposures: SBE) the insert shows more graphic details, (C) *T. viride* extract (single exposure: HB/TV, multiple exposures: TVE), or (D) *W. sebi* extract (single exposure: HB/WS, multiple exposures: WSE). HDM = house dust mite extract. Symbols indicate (§) the lowest significant dose compared to control (0 μg dose), (a) indicates significant versus other treatments within dose and (b) indicates significant versus single exposure within dose ($p < 0.05$). Error bars represent standard error of the mean. Control, $n = 12$; multiple exposures, $n = 5-6$; single exposure, $n = 4$.

demonstrated various levels of proteolytic activity. SBE demonstrated higher levels of protease activity than those of the trypsin control ranging from 96% of trypsin activity at 0 μg/ml to 446% at 20 μg/ml. The proteolytic activity of the other extracts were 97–196% for CCE, 97–71% for TVE, 98–22% for WSE, and 100–25% for HDM (Table 2).

Discussion

The goal of these studies was to determine the allergenic potential of selected G1 and G2 molds categorized by Vesper et al. (2006) compared to HDM (a prototypical causal agent for allergic asthma) in a dose-response manner.

Allergic responses induced by these mold extracts demonstrated differences in both the dose required to induce the response (Table 3) and the response magnitude (Table 4). In this study, the G1 molds (*T. viride*, *S. brevicaulis*, and *W. sebi*) had a more limited capacity to induce allergic responses requiring higher doses and less robust or no response compared to HDM. Additionally, airway responses, following a single exposure to TVE suggests that this mold may cause airway irritation and possibly exacerbate asthma symptoms. However, the *W. sebi* extract did not induce allergic responses at any dose tested in this BALB/c mouse model.

Cladosporium cladosporioides types 1 and 2, the only G2 mold assessed in these studies, appears to induce allergic/asthma-like responses at lower doses than the G1 molds

studied. In fact, CCE exposures induced responses similar to that of HDM exposures. In addition to responses characteristics of allergic asthma (elevated total IgE, increased total and inflammatory cells), significant airway responses were induced during Mch challenge.

Some of the differential responses among the fungi may be due to differences in the quality or quantity of the “allergens” present in these protein extracts. Kurup et al. (2001) showed that individual *Aspergillus fumigatus* allergens had different capacities to enhance IgE, eosinophils, and AHR in murine model of allergic asthma. That study also demonstrated that individual allergens can cumulatively affect the pathogenesis of *A. fumigatus*-induced allergic asthma.

Furthermore, protease activity has been shown to play an important role in allergic responsiveness. Kheradmand et al. (2002) demonstrated that protease activity could override airway tolerance and thus allow allergy induction. This may be caused by epithelial cell damage (Kauffman, 2003a) which can facilitate allergy induction by allowing allergens through the epithelial surface and/or by inducing immune mediators such as cytokines and prostaglandin E2 (Asokanathan et al., 2002; Kauffman, 2003b). Another mechanism could be the amplification of allergic inflammatory responses through protease-activated receptors on epithelial cells (Reed & Kita, 2004). In these studies, SBE had the highest level of protease activity and HDM and WSE the lowest. CCE, with the second highest level of protease activity (195.78% of trypsin control protease activity), was the most robust allergen and HDM

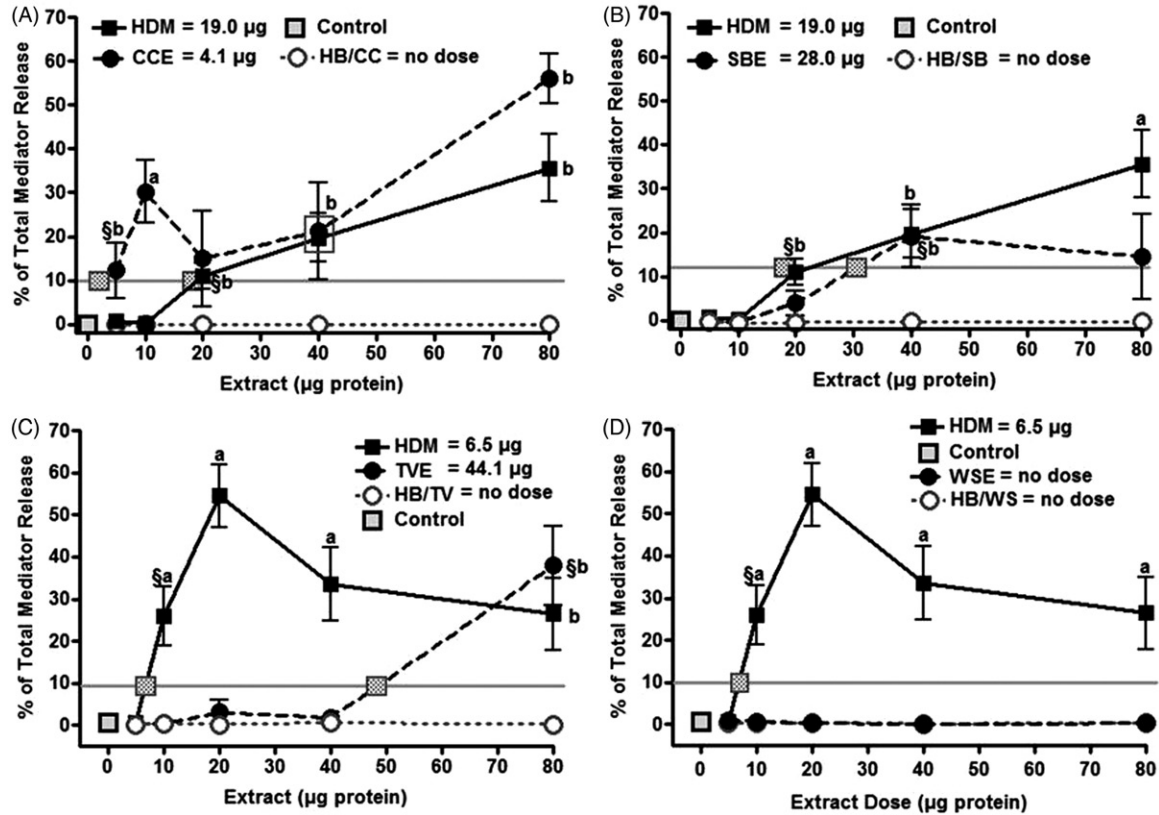


Figure 7. Extract-specific IgE assessed by RBL assay following a single or multiple exposures to (A) *C. cladosporioides* extract (single exposure: HB/CC, multiple exposures: CCE), (B) *S. brevicaulis* extract (single exposure: HB/SB, multiple exposures: SBE), (C) *T. viride* extract (single exposure: HB/TV, multiple exposures: TVE), or (D) *W. sebi* extract (single exposure: HB/WS, multiple exposures: WSE). Shaded box indicates the calculated dose that results in 10% of total mediator release stated in the legend. HDM = house dust mite extract. Symbols indicate (§) the lowest significant dose compared to Control (0 µg dose), (a) indicates significant versus other treatments within dose and (b) indicates significant versus single exposure within dose ($p < 0.05$). Error bars represent standard error of the mean. Control, $n = 12$; multiple exposures, $n = 5-6$; single exposure, $n = 4$.

Table 2. Extract protease activity compared to trypsin protease activity control.

Extract concentration	Trypsin FIU	HDM		CCE		SBE		TVE		WSE	
		FIU	% Tryp	FIU	% Tryp	FIU	% Tryp	FIU	% Tryp	FIU	% Tryp
0	403.64 (5.28)	404.97 (2.83)	100.33	390.14 (8.07)	96.66	386.59 (5.19)	95.78	392.33 (5.53)	97.20	395.22 (5.28)	97.91
0.5	801.70 (3.25)	423.89 (2.83)	52.87	657.42 (9.19)	82.00	987.29 (13.60)	123.15	523.03 (2.17)	65.24	456.87 (6.04)	56.99
1	925.73 (8.59)	436.26 (4.22)	47.13	868.58 (7.14)	93.83	1475.47 (11.00)	159.39	587.36 (9.03)	63.45	477.31 (12.13)	51.56
5	1416.55 (9.11)	481.80 (1.94)	34.01	1788.79 (16.27)	126.28	4095.09 (8.00)	289.09	919.78 (20.79)	64.93	489.88 (2.34)	34.58
10	1774.89 (33.62)	512.97 (2.61)	28.90	2566.66 (25.63)	144.61	6488.43 (71.60)	365.57	1183.67 (7.18)	66.69	504.92 (9.05)	28.45
20	2197.10 (30.30)	548.28 (7.89)	24.95	4301.55 (50.88)	195.78	9789.35 (71.40)	445.56	1551.38 (5.52)	70.61	491.60 (10.63)	22.38

Presented as fluorescence units [FIU (standard deviation)] and percent of trypsin activity control (% Tryp).

Table 3. Summary: lowest dose (microgram extract protein) that induced a significant response compared to 0 µg dose control.

Extract No. of exposures	Study I					Study II				
	HDM	CCE		SBE		HDM	TVE		WSE	
		4X	1X	4X	1X		4X	1X	4X	1X
BALF total cells ($\times 10^4$ cells/ml)	5 ^a	5	5	20	ns ^b	5	20	80	ns	ns
BALF neutrophil ($\times 10^4$ cells/ml)	5	5	5	ns	ns	5	80	40	ns	ns
BALF eosinophil ($\times 10^4$ cells/ml)	5	5	ns	20	ns	5	40	ns	ns	ns
Serum total IgE (ng/ml)	80	10	ns	ns	ns	5	40	80	ns	ns
Antigen-specific IgE (% mediator release)	20	5	ns	40	ns	5	80	ns	ns	ns
Log dose for 10% mediator release ^c	19.0	4.1	ns	28.0	ns	6.5	44.1	ns	ns	ns
Mch challenge (AUC)	40	10	ns	80	ns	40	80	40	ns	ns

^aDose (microgram extract protein) resulting in a significant response increase compared to 0 µg dose control.

^bns = no significant dose.

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

Table 4. Summary: average maximum response. The maximum response occurred at the 80 µg extract dose unless noted otherwise.

Extract No. of exposures	Study I						Study II					
	HDM 4X	CCE		SBE		Control	HDM 4X	TVE		WSE		Control
		4X	1X	4X	1X			4X	1X	4X	1X	
BALF total cells ($\times 10^4$ cells/ml)	88.61	122.08	58.66	30.31	6.42 ^a	5.11	97.95 ^b	88.89	36.16	9.41 ^a	8.60 ^a	6.56
BALF neutrophil ($\times 10^4$ cells/ml)	20.58	18.80	50.66	1.90 ^c	0.93	0.11	26.90	4.10	24.80	0.50 ^a	1.70 ^a	0.18
BALF eosinophil ($\times 10^4$ cells/ml)	39.01	67.90	0.05	13.10	0.83	0.02	50.90 ^b	33.60	1.30	1.40 ^a	0.10	0.05
Serum total IgE (ng/ml)	2528.4	18824.0	1183.3	2034.7	1005.7	867.4	3106.5 ^c	6692.2	1848.2	1400.5	1210.4 ^c	807.3
Antigen-specific IgE (% mediator release)	35.62	56.02	-0.06	19.16 ^c	-0.44 ^a	-0.12	54.55 ^b	37.97	0.65 ^c	1.04 ^a	0.50 ^b	0.80
Log dose (µg) for 10% mediator release ^d	19.0	4.1	nd ^c	28.0	nd	nd	6.5	44.1	nd	nd	nd	nd
Mch challenge (AUC)	87.33	70.56 ^c	36.91 ^c	58.49	30.60 ^b	27.71	30.85	32.22	32.18 ^c	22.70 ^a	26.70 ^a	19.01

^aAverage maximum response occurred at 40 µg extract dose level.^bAverage maximum response occurred at 20 µg extract dose level.^cAverage maximum response occurred at 5 µg extract dose level.^dCalculated dose that would induce sufficient antigen-specific IgE resulting in 10% of total mediator release in RBL assay.^end = no dose could be calculated to induce a 10% of total mediator response level.

(24.95% of trypsin) second lowest. Therefore, in these studies the role of protease activity in allergy induction is unclear. Additional non-antigen-specific inflammatory responses to the fungal extract could contribute to differences in allergic response induction. Both CCE and TVE exposed mice had a neutrophilic inflammatory response following a single exposure. The ability of these fungal extracts to induce a prominent neutrophilic inflammatory response even in naïve individuals could impact their potential to induce and/or exacerbate allergic asthma (Fahy et al., 1995; Jatakanon et al., 1999).

Co-exposure to endotoxin is another factor that may influence allergic responsiveness. In our studies, the levels of endotoxin ranged from 0.046 to 0.138 EU in 80 µg of extract protein which equates to 0–0.03 ng endotoxin (Table 1). These endotoxin levels are well below the levels expected to have an impact on allergic responses (Delayre-Orthez et al., 2004; Kim et al., 2007; Kitz et al., 2006; Sohy et al., 2006), suggesting that the endotoxin levels in these extracts had little if any effect on the response outcome of these studies.

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) and 2 µg *Der I* allergens/g dust was sensitizing for atopic children (Kuehr et al., 1994). 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.

In evaluating the results of the current study, it is important to assess the relevance of the exposure doses. The sensitizing exposure threshold dose like the one defined for HDM is not available for most allergens including molds/fungi. More recently, home assessment studies have identified (speciated) and quantified various molds in house dust using MSQPCR (Haugland et al., 2002; 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.

The relative allergenicity of the mold extracts compared to HDM was assessed 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 the influx of inflammatory cells (neutrophils and eosinophils) into the lungs. The allergic impact of the mold extract exposures was different for each mold. Multiple exposures were required to induce increased BALF eosinophil counts and serum antigen-specific IgE. It must be emphasized that regardless of the allergenic potential of an agent, exposure levels and sensitization thresholds are critical factors in allergic disease development. At this time, human exposure levels and sensitization thresholds are unknown for most allergens including molds/fungi. However, as exposure assessment methods improve, knowledge regarding exposure risk including potency will improve our understanding of healthy indoor environments.

Conclusions

These studies demonstrate the complex nature of molds and their differing capacities to induce a variety of adverse health effects associated with allergic asthma. Although these studies were not designed to address asthma exacerbation, some acute exposure responses (CCE, TVE BALF neutrophils and TVE Mch challenge) suggest that possibility for certain molds. Additionally, the data suggest a threshold dose for the induction of allergic responsiveness. At this time, human exposure levels and sensitization thresholds are unknown for most allergens including molds/fungi. Current methodology requires that assessing the allergic/asthmatic potential of molds needs to be performed on an individual basis. These studies along with our previous studies with *S. chartarum* (G1) (Chung et al., 2010; Viana et al., 2002) and *P. chrysogenum* (G2) (Chung et al., 2005; Ward et al., 2010) suggest that the G1/G2 categorization is not indicative of allergy induction potential. However, they do not preclude the utility of this categorization for the identification of

unhealthy building dampness. It should be noted that one effect of remediating building water-damage and dampness, which has been shown to reduce asthma symptoms (Kercsmar et al., 2006), is the control of mold/fungi.

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Declaration of interest

This research article has been reviewed by the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflects the views and policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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