

RESEARCH ARTICLE

The relative allergenicity of *Stachybotrys chartarum* compared to house dust mite extracts in a mouse model

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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. The authors compared the ability of the fungus *Stachybotrys chartarum* (SCE) 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 µg) 4 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 that induced a significant response compared to control (0 µg) and the linear regression slope analysis across the dose range. SCE induced a more robust response than HDM for BALF some inflammatory cells (macrophage and neutrophils), whereas HDM induced more robust BALF lymphocyte and eosinophil responses. Although SCE induced a more robust serum total immunoglobulin E (IgE) response than did HDM, the induction of a similar response in a functional, antigen-specific IgE assay required approximately twice as much SCE as HDM. Even though SCE demonstrates the ability to induce allergic responses in the mouse model, considering the importance and relevance of eosinophil, lymphocyte, and antigen-specific IgE in allergic airway disease, it is concluded that HDM is more potent than SCE in the induction of allergic responses. These data suggest a threshold dose for SCE allergy induction. Furthermore, in damp water-damaged environments, exposure to *S. chartarum* might easily exceed the sensitization threshold for a susceptible population.

Keywords: Allergy; house dust mite; mold; mouse model; *Stachybotrys chartarum*

Abbreviations: BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; HBSS, Hank's balanced salt solution; IA, intratracheal aspiration; IgE, immunoglobulin E; IgG, immunoglobulin G; PBS, phosphate-buffered saline; SCE, *Stachybotrys chartarum* extract; HDM, house dust mite; LDH, lactate dehydrogenase; RBL cells, rat basophilic leukemia cells; IOM, the Institute of Medicine.

Introduction

Molds are ubiquitous in the environment. An Institute of Medicine (IOM) report (NRC, 2000) concluded that an association exists between damp buildings and exacerbation of asthma symptoms. Although the IOM report states that there is currently insufficient evidence to conclude that molds induce asthma, a recent review (Denning et al., 2006) described epidemiological evidence of an association between fungal exposure and asthma severity.

Building-related illnesses are often linked to dampness, which promotes fungal growth (Kildeso et al., 2003). *Stachybotrys chartarum*, a cellulytic mold found in water-damaged environments, has been associated with

building related illness (Hintikka, 2004) as well as airway inflammation (Leino et al., 2006; Yike et al., 2005). These and other adverse health effects attributed to *S. chartarum* exposures were recently reviewed by Pestka et al. (2008). Several laboratories have identified *S. chartarum* proteins that are reactive with human sera immunoglobulin E (IgE) (Raunio et al., 2001; Barnes et al., 2002; Xu et al., 2007; Karkkainen et al., 2004). Furthermore, Karkkainen et al. (2004) determined that of the eight IgE-reactive *S. chartarum* proteins, more than 80% of the human sera from exposed individuals (<50% of control sera) were reactive with three of these proteins (33-, 48-, and 50-kDa proteins). Additionally, these sera were also reactive with the 48- and 50-kDa proteins

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from seven other molds, suggesting shared antigens. Peptide analysis determined that the 48-kDa protein demonstrated homology with fungal cellulases (Karkkainen et al., 2004). *S. chartarum* has also been shown to be capable of inducing IgE-mediated diseases (including asthma) in animal models (Barnes et al., 2002; Karkkainen et al., 2004; Viana et al., 2002).

Although animal studies have demonstrated the ability of some molds to induce allergic and asthma-like responses, few studies have compared molds based on their relative ability or potential to induce allergy. Furthermore, a comparison of mold extract responses to a well-characterized allergen (house dust mite) may indicate the relative risk of exposure to these agents.

Our study goal was to determine differences in the allergic responses induced by *S. chartarum* extracts (SCE) compared to house dust mite extract (HDM), a known inducer of allergic asthma (NRC, 2000), using a mouse model of allergic asthma.

Materials and methods

Animals

Fifty-day-old female BALB/c mice (Charles River, 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 (AAALAC)-accredited animal facility. 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. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory (NHEERL), US Environmental Protection Agency (EPA). Mice were allowed to acclimate 1 week prior to the start of the experiment.

Fungal antigen preparation

Five *S. chartarum* isolates (JS5807 [ATCC accession no. 201864], JS5816 [201867], JS5817 [201211], JS5818 [201212], and JS6301 [201870]) were originally obtained from residences in Cleveland, OH (Etzel et al., 1998). These isolates were chosen for this study in order to be consistent with earlier studies from our laboratory (Viana et al., 2002). The individual isolates were grown on nylon filters resting on sheep's blood agar or cellulose agar plates at 23°C for 1 month (Viana et al., 2002). The fungal mass (mycelium and spores) of each isolate was extracted as described in Ward et al. (1998). Briefly, the isolates were 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, Rockville, MD) + 0.05% (*v/v*) Tween 80 (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, Beverly, MA) using YM3 membranes (molecular weight > 3000 Da cutoff) and filter sterilized with a 0.2-μm syringe filter. Each individual crude antigen extract was assayed for total protein concentration using a Pierce BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard according to manufacturer's procedures. Equal protein concentrations of each isolate were combined resulting in the mixture designated *S. chartarum* extract (SCE). SCE was stored at -80°C until use. Endotoxin level of the extract was measured using a *Limulus amoebocyte* lysate test kit (BioWhittaker, Walkersville, MD); the level at the highest dose (80 μg) was 0.03 Endotoxin Units (EU).

Lyophilized whole-body extracts of house dust mites (HDM) *Dermatophagoides farinae* and *D. pteronyssinus* purchased from Greer Laboratories (Lenoir, NC) were rehydrated in HBSS to 3 mg/ml, mixed equally and stored in aliquots at -0°C until use. Endotoxin level of the highest dose (80 μg) was 0.27 EU.

Experimental design

Six mice/dose were exposed to 2.5, 5, 10, 20, 40, or 80 μg of SCE or HDM in HBSS (total volume of 50 μl) by intratracheal aspiration (IA) 4 times over a 4-week period, as previously described (Ward et al., 2000). An additional group of 12 mice was exposed to HBSS only as a control. Briefly, mice were anesthetized by inhalation of a mixture of isoflurane and 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 time line (Figure 1) is based on the immunology paradigm that the primary immune response is approximately 7–14 days following the initial exposure (days -28 to -14 in our experimental protocol) and approximates the sensitizing phase of allergy induction. The second exposure in our protocol is 14 days (day -14) after the first thus is on the boundary between sensitization and challenge. However, the last two exposures at days -7 and 0 are challenge exposures. Serum and bronchoalveolar lavage fluid (BALF) were collected 3 days following the final exposure. BALF was assessed for lactate dehydrogenase (LDH), total protein, and total cell

EXPERIMENTAL TIMELINE:

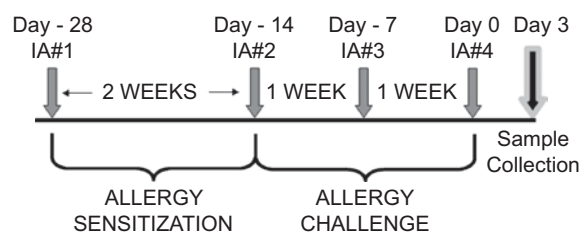


Figure 1. Experimental design time line. Mice were administered SCE, HDM, or HBSS (control group) by intratracheal aspiration (IA) 4 times over a 4-week period. Allergy sensitization and challenge phases are indicated. Blood and BALF were collected 3 days after the final exposure.

and differential cell counts. Serum was assessed for total and SCE- or HDM-specific IgE. The responses of allergen extract-treated mice were compared to results obtained from HBSS-treated control animals.

Bronchoalveolar lavage (BAL) and blood collection

Blood and bronchoalveolar lavage fluid (BALF) samples were collected as previously described (Ward et al., 1998). 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 $100 \times g$ for 15 min at 4°C . An aliquot of the supernatant was stored at 4°C for total protein and lactate dehydrogenase activity assay 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, Miami, FL). Additionally, 100–150 μl of resuspended cells were adhered onto glass slides at 200 rpm for 10 min using a Cytospin 2 centrifuge (Shandon, Pittsburgh, PA). The BALF cells on glass slides were stained with Wright-Giemsa (Fisher Scientific, Pittsburgh, PA) on a Hema-Tek 2000 slide stainer (Miles, Elkhart, IN), 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 (St. Louis, MO). Additionally, the BALF samples were assayed for LDH activity using a commercially prepared kit and controls from Sigma Chemical. 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 enzyme-linked immunosorbent assay (ELISA) was performed as previously described by Chung et al. (2005). Purified monoclonal anti-mouse IgE (Pharmingen, San Diego, CA) in phosphate-buffered saline (PBS), 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) was the detection enzyme with 3,3',5,5'-tetramethyl benzidine (TMB) substrate (DAKO, Carpinteria, CA). Optical density was read on a Spectramax 340PC Plate Reader (Molecular Devices, Menlo Park, CA) at a wavelength of 650 nm. Softmax Pro version 2.6.1 (Molecular Devices) 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/well (ATCC, Rockville, MD) 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 control) 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 extract or buffer alone (no antibodies). 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) at a wavelength of 405 nm and data collected by Softmax Pro software (version 2.6.1; Molecular Devices). Data are presented as percent of total mediator (β -hexosaminidase) release following subtraction of spontaneous release. Additionally, the extract dose resulting in 10% of total release was calculated by interpolation.

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 $\mu\text{g}/\text{ml}$) 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 (Spectra Max, Gemini XS; Molecular Devices) using a filter with excitation = 590 ± 10 nm, emission = 645 ± 20 nm. Data are reported as fluorescence units and % of trypsin control.

Statistical analysis

The data were analyzed using a Dunnett's comparison procedure. Groups were formed by exposure-dose combinations and then simultaneously compared to the controls. The level of significance was set at .05.

To evaluate the responses across the dose range, an analysis of linear regression slope was performed. Assuming linearity over the dose range (2.5–80 μg of extract), a linear model was used to simultaneously fit the individual regression lines for the extracts, using data from the responses to six doses. The slopes, as a measure of potency, were compared using a pair-wise subtest (*F* test) of the overall model. For all analyses, the level of significance was set at $p < .05$. No adjustment was made for multiple comparisons because of the increase in the type II error rate.

The analysis of antigen-specific IgE (RBL assay) data consisted of two parts. Part one fit linear regression lines to the various exposures and compared the estimated slopes. Part two estimated the dose at which a 10% of total mediator release occurred. This was accomplished through linear interpolation between responses at consecutive doses bracketing the 10% value.

Results

Total protein and lactate dehydrogenase (LDH) levels

Elevations in BALF total protein are indicative of pulmonary vascular leakage (pulmonary edema). The lowest dose at which mice showed a significant increase in total protein levels compared to the 0 μg dose control mice was 20 μg of HDM or 10 μg of SCE ($p < .05$) (Figure 2A). However, the responses at these dose levels were not significantly different from each other nor were there significant differences between HDM and SCE responses at any given dose.

Elevated LDH activity in BALF is indicative of nonspecific cellular damage. The lowest dose at which mice showed a significant increase in LDH activity compared to the 0 μg dose controls was 20 μg of HDM (Figure 2B). Although SCE-exposed mice did not have a significant change in BALF LDH activity compared to 0 μg dose, it is clear that the variability for this endpoint among the SCE exposed mice affected the outcome of this analysis. Furthermore, there were no significant differences between the responses to HDM and SCE at any given dose level. However, linear regression slope analysis did find a significant difference between the dose response profiles of the two extracts.

These data suggest that the risk for developing lung edema is similar following exposure to either HDM or SCE, but HDM appears to have a greater potential to induce nonspecific cellular injury.

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. Although there were differences in the cellular influx profile, as well as the dose at which the response seemed to plateau, generally, both HDM and SCE exposures induced dose-dependent BALF total and differential cell count increases. The lowest dose of either HDM or SCE

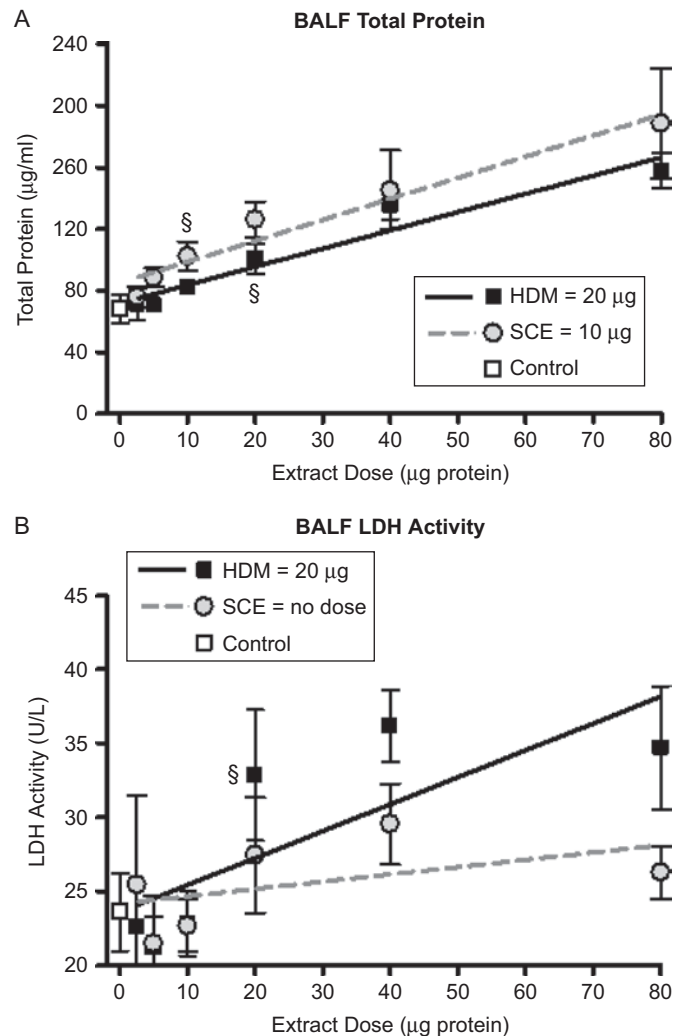


Figure 2. Total protein (A) and LDH activity (B) in BALF of HDM- or SCE-exposed mice collected 3 days after the final exposure. § indicates the lowest significant dose compared to HBSS (0 dose control) ($p < .05$). Error bars represent standard error of the mean. $n = 5-6$.

that induced a significant increase in the BALF total and macrophage cell counts compared to the 0 dose control was 5 μg of extract per exposure. The total cell counts were $11.00 \pm 1.43 \times 10^4$ cells/ml, with macrophages representing 84.56%, and $12.76 \pm 1.62 \times 10^4$ cells/ml, with macrophages representing 86.34%, respectively ($p < .05$) (Figure 3A and B). However, the SCE macrophage counts were significantly elevated compared to HDM at the 10, 20, and 40 μg dose levels, but did not continue to increase after the 40 μg dose level. Additionally, the linear regression slope analysis of the macrophage counts demonstrated a significant difference between the two extracts across the dose range.

Ten micrograms of extract was the lowest dose of either HDM or SCE that induced significant increases in BALF lymphocyte and eosinophil counts compared to the 0 dose controls ($p < .01$) (Figure 3C and D). HDM-exposed mice had significantly elevated lymphocyte and eosinophil counts compared to those of the SCE-exposed mice at the 80 μg dose level. Thus the linear regression slope analysis

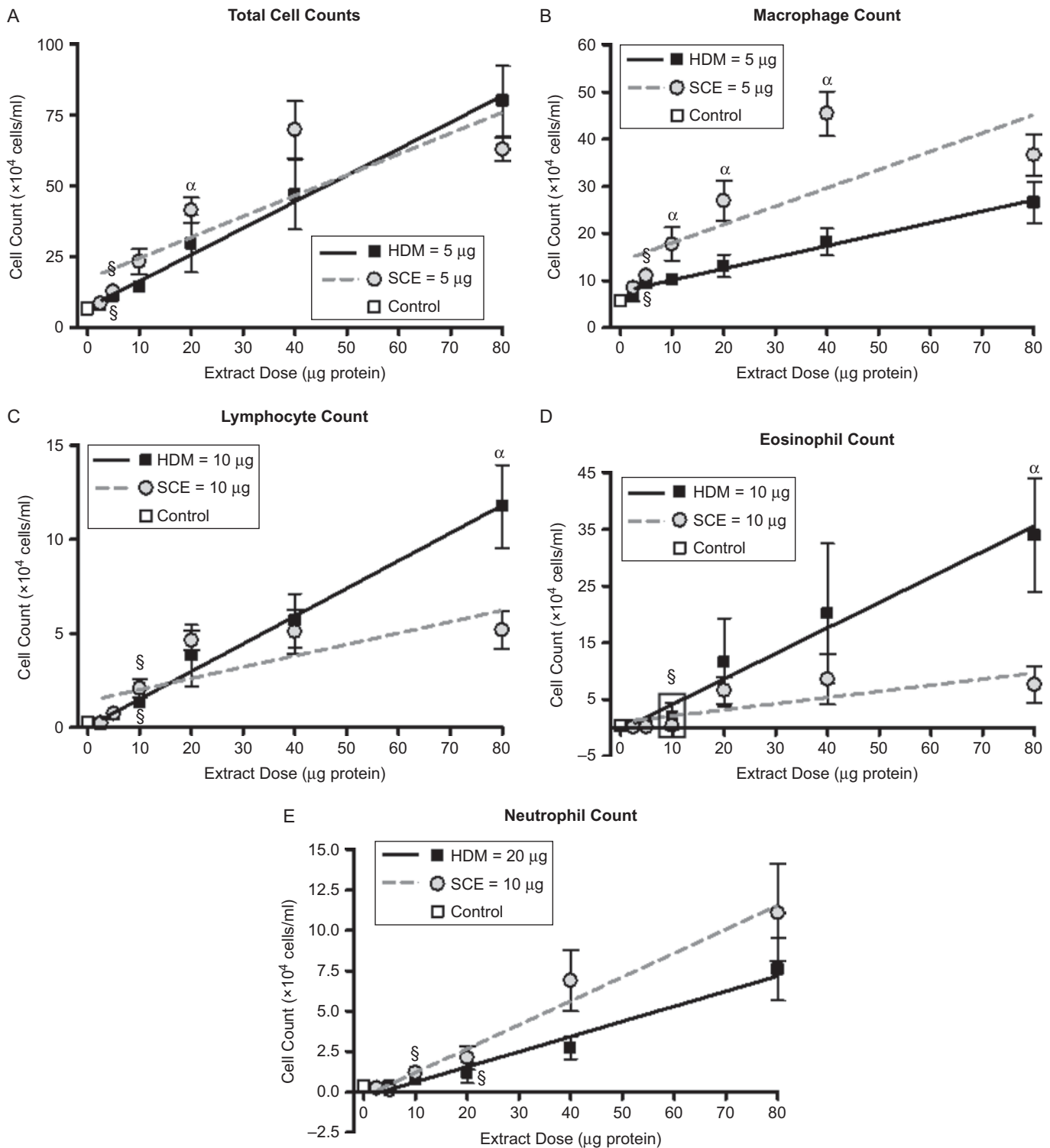


Figure 3. Total cell counts (A) and differential cell counts, macrophage (B), lymphocyte (C), eosinophil (D), and neutrophil (E), in BALF of HDM- or SCE-exposed mice collected 3 days after the final exposure. § indicates the lowest significant dose compared to HBSS (0 dose control) ($p < .05$). α indicates statistically significant differences within the dose. Error bars represent standard error of the mean. $n = 5-6$.

demonstrated a significant difference across the dose range for both cell types.

The lowest dose of HDM and SCE that induced a significant elevation of BALF neutrophil counts compared to 0 μg dose controls was 20 μg ($1.17 \pm 0.64 \times 10^4$ cells/ml) and 10 μg ($1.19 \pm 0.42 \times 10^4$ cells/ml) ($p < .05$)

(Figure 3E). Additionally, the linear regression slope analysis demonstrated that SCE induced a steeper increase in neutrophil counts than that induced by HDM across the dose range.

In a separate study, mouse BALF was collected 2 days after the mice received a single 20 μg dose of extract. HDM

Table 1. Inflammatory cell counts: BALF neutrophil and eosinophil cell counts 2 days after a single exposure

Treatment	Neutrophils ($\times 10^4$ cells/ml)	Eosinophils ($\times 10^4$ cells/ml)
HBSS (0 μ g)	0.19 (0.08)	0.01 (0.00)
HDM	19.24 (2.63)	0.83 (0.28)
SCE	1.37 (0.33)	0.14 (0.04)

Note. The HBSS response for the multiexposure counts is the average of all 0 μ g animals in the study. Data shown are the average (standard error of the mean).

induced a larger influx of both neutrophils and eosinophils compared to SCE (Table 1).

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 both HDM- and SCE-treated mice (Figure 4A). The lowest dose that induced a significant increase in serum total IgE compared to 0 μ g dose controls was 20 μ g of HDM or 10 μ g SCE ($p < .05$). Generally, the level of serum total IgE was higher in SCE-treated mice than in HDM-treated mice, but this difference was significant only at the 2.5 μ g dose level. However, a significant difference was found between the two extracts when the linear regression slope analyses were compared ($p < .05$).

Analysis of BALF IgE levels did not show any significant increases when compared to the 0 μ g dose nor between the extracts at any dose (data not shown).

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. A significant difference was found when the extract dose-response curve linear regression slope analyses were compared. The lowest extract dose that induced a significant increase in mediator release compared to the 2.5 μ g dose was 20 μ g of HDM and 40 μ g SCE (Figure 4B).

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 (Figure 4C). The calculated dose of HDM required to achieve 10% of total mediator release was 13.17 μ g. However, it required slightly more than twice as much SCE (27.79 μ g) to achieve the same result.

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. Protease activity was similar for HDM and SCE at all concentrations, ranging from 25% to 53% and 30% to 56% of trypsin proteolytic activity, respectively (Table 2).

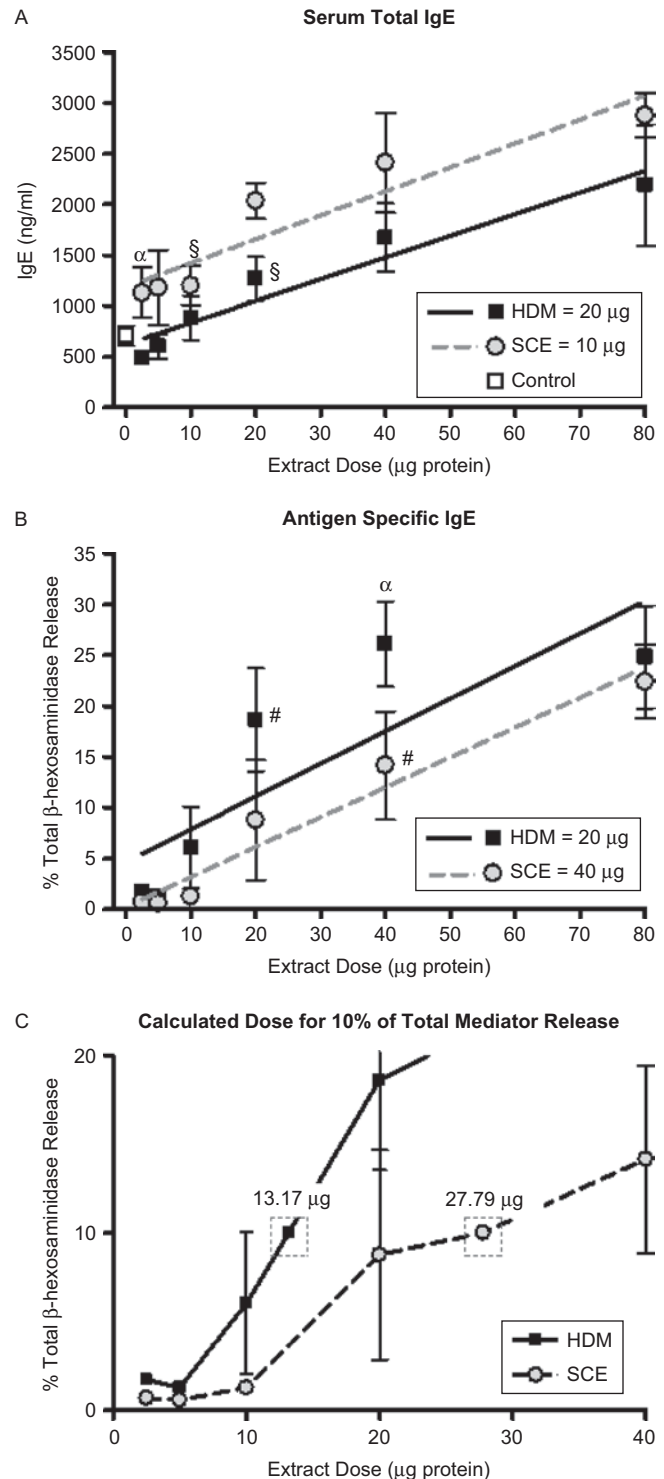


Figure 4. (A) Serum total IgE levels of HDM- or SCE-exposed mice collected 3 days after the final exposure. (B) Dose-response and linear regression line for percent of total β -hexosaminidase release from RBL cells (index of antigen-specific IgE) after incubation with serum (1:4 dilution) obtained from HDM- or SCE-exposed mice 3 days after the final exposure. (C) Extract doses, which resulted in 10% of total β -hexosaminidase release, were calculated by interpolation. § indicates the lowest significant dose compared to HBSS (0 dose control). # indicates the lowest significant dose compared to 2.5 μ g dose (no 0 dose tested in RBL assay) ($p < .05$). α indicates statistically significant differences within the dose. Error bars represent standard error of the mean. $n = 5-6$.

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

Extract concentration ($\mu\text{g/ml}$)	Trypsin	HDM		SCE	
	Fluorescence units (SEM)	Fluorescence units (SEM)	% of Trypsin	Fluorescence units (SEM)	% of trypsin
0.0	403.64 (3.05)	404.97 (1.63)	100.33	376.30 (5.21)	93.23
0.5	801.70 (1.88)	423.89 (1.64)	52.87	446.48 (2.51)	55.69
1.0	925.73 (4.96)	436.26 (2.44)	47.13	448.71 (1.36)	48.47
5.0	1416.55 (5.26)	481.80 (1.12)	34.01	457.95 (3.70)	32.33
10.0	1774.89 (19.41)	512.97 (1.51)	28.90	541.53 (0.34)	30.51
20.0	2197.10 (17.49)	548.28 (4.55)	24.95	660.83 (10.34)	30.08

Discussion

Previous studies in our laboratory (Viana et al., 2002) and the study presented here have found that *S. chartarum* extracts have the capacity to induce allergic and asthmatic responses in a mouse model. In the present study, the relative allergenicity of the *S. chartarum* extracts compared to HDM was assessed in a dose-response manner and was evaluated 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 3). Although near equivalent amounts of HDM and SCE induced elevated responses with regard to inflammation parameters, the critical importance of antigen-specific IgE, eosinophils, and lymphocytes in allergic airway disease pathogenesis leads us to conclude that HDM is more potent than SCE with regard to allergy induction.

Elevated serum total IgE level has been used as a generalized marker of atopy, with antigen-specific IgE antibodies indicating the allergen trigger. Interestingly, in this study, mice exposed to SCE demonstrated slightly higher but nonsignificant total IgE increases compared to mice exposed to the same concentrations of HDM. Despite this difference, to achieve a similar result in the functional IgE assay required approximately twice as much SCE as HDM (Figure 4C). The reason for this discrepancy might be a "bystander" effect on total IgE production. Moss (2005) has previously demonstrated that an interleukin (IL)-4-dependent elevation of nonspecific IgE was induced by the pathogenic fungus *Aspergillus fumigatus*, the causal agent for allergic bronchopulmonary aspergillosis (ABPA). Furthermore, this total IgE elevation in the ABPA model has been correlated with the fungus' ability to stimulate antigen-independent costimulatory molecules on T and B cells (Barrios et al., 2005).

With the exception of neutrophil influx, equivalent amounts of HDM and SCE induced significant increases of BALF total and differential cells counts compared to the 0 dose control. However, linear regression slope analysis indicated significant differences in the slopes across the dose range for the differential cell counts. SCE induced a more robust macrophage and neutrophil response than did HDM, suggesting a more nonspecific inflammatory response. However, HDM induced a more robust lymphocyte and eosinophil response than did SCE suggesting a more potent allergic response.

Table 3. Summary of endpoints characteristic of human allergic/asthma^a.

Source	Endpoint	Relative potency	
		Lowest dose for significant response ^b (response magnitude)	
		HDM	SCE
BALF	Lymphocyte count ($\times 10^4$ cells/ml)	10 (1.33 $\pm$ 0.21)	= 10 (2.08 $\pm$ 0.50)
	Neutrophil count ($\times 10^4$ cells/ml)	20 (1.17 $\pm$ 0.64)	> 10 (1.19 $\pm$ 0.42)
	Eosinophil count ($\times 10^4$ cells/ml)	10 (2.02 $\pm$ 0.42)	= 10 (2.40 $\pm$ 0.73)
Serum	Total IgE (ng/ml)	20 (880.71 $\pm$ 219.28)	> 10 (1199.60 $\pm$ 190.36)
	Antigen-specific release ^b (% total mediator release)	20 (18.64 $\pm$ 5.08)	< 40 (14.15 $\pm$ 5.31)
	Antigen-specific IgE ^c ($\mu\text{g extract}$)	13.17	< 27.79

^aBased on the lowest dose that induced a significant response compared to the 0 μg dose and the magnitude of the response at that dose. For antigen-specific IgE, dose comparison was made to the 2.5 μg dose.

^bDose ($\mu\text{g extract}$) resulting in a significant increase over 0 μg dose control at $p < .01$.

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

A single exposure to either extract (20 μg) 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). Because the single- (Table 1) versus multiple- (Fig 3E) exposure experiments were performed in separate studies and samples were collected at 48 versus 72 h following allergen exposure, only speculative conclusions may be drawn. Nonetheless, it appears that depending upon the extract, multiple exposures may have no apparent effect on neutrophil levels (SCE) or may blunt the response (HDM). In contrast, multiple exposures to either of these extracts induce a more robust eosinophil influx (Table 1 and Figure 3D).

Protease activity has been shown to play a role in allergic responsiveness (Kheradmand et al., 2002) through epithelial cell damage (Kauffman, 2003) or protease-activated receptor amplification of allergic inflammatory responses

(Reed and Kita, 2004). Therefore differences in protease activity might affect the differential responses seen between extracts. However, SCE and HDM demonstrated similar protease activity at all concentrations evaluated, suggesting that this is not a major distinguishing factor between the two with regards to allergenic potency.

Another factor that may influence allergic responsiveness is coexposure to endotoxin. In human studies exposure to low levels of endotoxin (LPS, 2 µg; domestic environmental level) before allergen challenge did not have a significant effect on immediate or late-phase bronchial responses to cat allergen nor eosinophilic inflammation (local and systemic) (Sohy et al., 2006). Furthermore, LPS challenge (100 µg cumulative dose) of healthy and asthmatic individuals induced a temporary increase in bronchial endothelial nitric oxide (eNO) in an asthmatic subgroup but otherwise asthmatic responses were not different from those of healthy individuals (Kitz et al., 2006). However, mixed results have been found with coexposure to endotoxin/LPS during sensitization in an ovalbumin allergy/asthma mouse model. One study found increased allergic responses when sensitization occurred in the presence of 100 ng LPS (Kim et al., 2007), whereas another found no significant effect with 10 ng LPS (Delayre-Orthez et al., 2004). However, administration of 1 ng LPS during challenge did increase BALF eosinophils, total cells, and IL-5 (Delayre-Orthez et al., 2004). In our study, the HDM extracts had the higher level of endotoxin (0.27 EU in 80 µg of extract protein) which equates to ~0.027–0.054 ng LPS. This is well below the levels expected to have an impact on allergic responses. This suggests that the endotoxin levels in these extracts had little if any effect on the response outcome in this study.

Additionally, differences between the extract-induced responses may result from differences in the quality or quantity of the 'allergens' in the extracts. As has been shown in murine model of allergic asthma, specific *A. fumigatus* allergens differ in their ability to induce IgE, eosinophils, and airway hyperresponsiveness and can differentially affect the pathogenesis of allergic asthma (Kurup et al., 2001). Therefore in evaluating the results of the current study, it is important to assess the relevance of the exposure dose. In the case of HDM, exposure to ≥2 µg *Der I*/g dust is a HDM sensitization risk factor for atopic children (Lau et al., 1989; Platts-Mills et al., 1992), but 80 µg *Der I* allergen/g dust was required to sensitize nonatopic children (Kuehr et al., 1994). Arbes et al. (2003) estimated that most US homes have detectable levels of HDM in beds, with 46.2% ± 2% of beds containing 2 µg HDM allergen/g dust and 24.2% ± 2.1% of beds having 10 µg/g of dust. In this study, HDM exposures of 10 µg contained 1.96 µg of *Der I* allergens, a dose near the proposed sensitization level for atopic children and within the range of US bed HDM concentration estimates (Arbes et al., 2003).

The sensitizing exposure threshold dose like the one defined for HDM is not available for most allergens including *S. chartarum* and other molds. However, recent home

assessment studies have identified and quantified various molds in the water-damaged homes of asthmatics compared to the homes of nonasthmatics in Cleveland, OH (Vesper et al., 2006). Assessment of the fungal content of house dust using mold-specific quantitative polymerase chain reaction (MSQPCR) (Haugland et al., 2002) demonstrated the presence of *S. chartarum* in 46% of the water-damaged homes. Although *S. chartarum* was present at low concentrations in water-damaged homes (648.07 cell equivalents/g dust geometric mean), these levels were still significantly increased compared to those found in non-water-damaged homes (334.71 cell equivalents/g dust geometric mean). In another study of Detroit homes (Vesper et al., 2008), no differences in *S. chartarum* (3 cell equivalents/mg dust) concentrations or prevalence (79% versus 61%) were found between asthmatic and nonasthmatic homes. In both studies, the concentrations of *S. chartarum* was low compared to the concentrations of many other fungi detected. These data, plus dose-response comparisons like the one presented in this study, may provide insight into sensitizing dose thresholds and exposure levels for *S. chartarum* and other molds.

The detergent industry provides an example of relative potency testing where both human and animal data are available. An evaluation of the relative potency of new detergent enzymes to the benchmark enzyme *Bacillus subtilis* subtilisin A (Alcalase) in a mouse model mirrored the exposure levels found to be safe for their human workforce (Sarlo et al., 1997; Robinson et al., 1998). Thus, the relative potency of detergent enzymes or other substances to induce allergic responses in an animal model may be useful in hazard identification for human allergy induction.

This study demonstrates the complexity of the responses induced by extracts that contain allergenic proteins. The eosinophil, lymphocyte, and antigen-specific IgE data are more relevant to allergy, and thus contribute to an overall greater potency of HDM despite a more robust BALF total cellular, macrophage, neutrophil, and serum total IgE response for SCE. Due to the importance of functional antigen-specific IgE, it is particularly noteworthy that approximately twice as much SCE as HDM was required to induce a similar response in a functional IgE assay, regardless of whether that response was assessed by lowest significant dose or by calculated dose to induce 10% of total mediator release. Therefore, based on these data we have concluded that HDM is a more potent allergen source than SCE.

Having said this, it must be stated that 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, human exposure levels and sensitization thresholds are unknown for most allergens, including molds/fungi. Importantly, the data presented in the current study suggest a threshold dose for the induction of allergic responsiveness to SCE. Furthermore, in damp water-damaged environments, exposure to *S. chartarum* might easily exceed the sensitization threshold for a susceptible subpopulation.

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Declaration of interest

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