

An Extract of *Stachybotrys chartarum* Causes Allergic Asthma-like Responses in a BALB/c Mouse Model

Michael E. Viana,* Najwa Haykal Coates,† Stephen H. Gavett,† MaryJane K. Selgrade,†
Stephen J. Vesper,‡ and Marsha D. W. Ward†¹

*Department of Anatomy, Physiological Sciences, and Radiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606; †National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, 86 T. W. Alexander Drive, MD92, Research Triangle Park, North Carolina, 27711; and ‡National Exposure Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268

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Environmental exposure to *Stachybotrys chartarum* has been associated with multiple adverse health effects in humans. The goal of this study was to assess soluble components of this fungus for their ability to cause an asthma-like response in a BALB/c mouse model. Five isolates of *S. chartarum* were combined and extracted to form a crude antigen preparation (*S. chartarum* extract 1 [SCE-1]). Female BALB/c mice were sensitized by involuntary aspiration of SCE-1 and subsequently reexposed at 2, 3, and 4 weeks. To distinguish immune from nonspecific inflammatory effects, mice were exposed to 3 doses of Hanks' balanced salt solution (HBSS) and a final dose of SCE-1; or to 4 doses of bovine serum albumin (BSA) as a negative control protein. Serum and bronchoalveolar lavage fluid (BALF) were collected before the fourth aspiration (Day 0), and at Days 1, 3, and 7 following the final exposure, and lungs were fixed for histopathological examination. SCE-1-exposed mice displayed increased BALF total protein on Days 0, 1, and 3 and increased lactate dehydrogenase (LDH) at Days 1 and 3 only, compared to HBSS controls. BALF total cell numbers were elevated on each day, and differential counts of BALF cells showed neutrophilia on Day 1, marked eosinophilia on all days, and increased numbers of lymphocytes at Days 1, 3, and 7. Serum and BALF total IgE levels were elevated at all days, and BALF IL-5 levels were greatly increased (7-fold) on Day 1. Mice exposed to a single dose of SCE-1 exhibited inflammatory responses but not allergic responses, while BSA-treated mice showed neither inflammatory nor allergic responses. Histopathology confirmed the biochemical findings. Barometric whole-body plethysmography was performed 10 min prior to (baseline) and one h following each aspiration exposure in a second group of mice, to assess immediate respiratory responses. Airway hyperresponsiveness to increasing concentrations of nebu-

lized methacholine (MCh) was assessed on Days 1 and 3 following the fourth aspiration exposure. Exposure to HBSS or BSA did not alter baseline enhanced pause (PenH) values or PenH following the aspiration exposures, nor did it cause an increase in airway responsiveness to MCh. Exposure to SCE-1 resulted in a 4.7-fold increase in PenH over baseline after the third exposure, increasing to 5.6-fold after the final exposure, and increased responsiveness to a 32 mg/ml MCh aerosol challenge. We conclude that multiple respiratory exposures to SCE-1 cause responses typical of allergic airway disease in this mouse model. However, BSA was nonallergenic and did not generate respiratory physiological responses when administered by aspiration.

Key Words: allergy; asthma; *Stachybotrys chartarum*; respiratory exposure.

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¹To whom correspondence should be addressed. Fax: (919) 541-4284. E-mail: ward.marsha@epa.gov.

Asthma is a complex chronic respiratory disorder characterized by pulmonary inflammation, mucus secretion, and intermittent airway constriction and airway hyperresponsiveness, resulting in symptoms of chest tightness, wheeze, and shortness of breath. The incidence of asthma is increasing: an estimated 14.6 million adults in the United States had current asthma during the year 2000 (CDC, 2001), resulting in an estimated economic burden of 12.7 billion dollars (U.S.) for 1998 (Weiss and Sullivan, 2001). Asthma is most prevalent in young children, with acute viral infections acting as a trigger of symptoms in approximately 85% of cases. Approximately half of these individuals stop wheezing by adolescence; however, children exhibiting the most severe manifestations of asthma usually have a clear allergic component, become symptomatic early in life, and continue through adolescence. Thus, in adults suffering from asthma, >90% are found to be allergic asthmatics (Holt *et al.*, 1999). It has been established that allergic asthma is a T cell-mediated immune response driven by allergenic peptide presentation to memory Th2 cells (Holt *et al.*, 1999), and is further characterized by a biphasic response composed of acute (IgE-mediated) and late-phase reactions thought to be mediated by the presence of Th2 cells, mast cells,

and eosinophils within the walls of the lower respiratory tract (Kobayashi *et al.*, 2000).

A variety of microscopic fungi, also known as molds, are known to cause a number of diseases in humans, ranging from frank colonization of tissues to immune responses in atopic individuals (Pieckova and Jesenska, 1999). The toxigenic mold *Stachybotrys chartarum* has been associated with a number of health effects, including acute pulmonary hemorrhage in infants (Elidemir *et al.*, 1999; Etzel *et al.*, 1998; Montana *et al.*, 1997; Novotny and Dixit, 2000; Tripi *et al.*, 2000), and sick-building syndrome (Cooley *et al.*, 1998; Mahmoudi and Gershwin, 2000). In addition, both anecdotal and published reports have associated exposure to this mold with asthma (Hodgson *et al.*, 1998). Previous research has focused on the effects of the potent trichothecene mycotoxins produced by *S. chartarum* (Croft *et al.*, 1986; Jarvis *et al.*, 1995, 1998; Sorenson *et al.*, 1987), and the recently described hemolytic protein stachylysin (Vesper *et al.*, 2001). However, the potential exists for genetically predisposed individuals inhabiting buildings or homes contaminated with *S. chartarum* to be directly exposed to aerosolized *S. chartarum* (whole or fragmented conidia and/or mycelium) and to develop allergic responses, resulting in or exacerbating existing respiratory disease. The goal of the current study was to assess the allergenic potential of *S. chartarum* in a controlled animal study.

Animal models have been used to study inflammatory and immunological responses of the respiratory tract to allergens, and to investigate airway hyperresponsiveness and inflammation. Initially, *in vivo* measurements of lung conductance and compliance following intravenous infusions of bronchoconstrictive agents (Martin *et al.*, 1988), and to aerosolized antigen (Renz *et al.*, 1992), were performed in anesthetized, tracheostomized, mechanically ventilated mice. However, the effect of the surgical procedure and anesthesia on respiratory physiological responses was not fully known, pulmonary responses to bronchoconstrictor agents administered by intravenous injection were not well defined, and the animals could only be subjected to one experimental measurement, being killed at its conclusion. To address these shortcomings, barometric whole-body plethysmography was developed, allowing measurement of airway responsiveness to aerosolized agents to be determined in unrestrained conscious mice (Travis *et al.*, 1982). This method allows repeated measurements to be performed on the same subject, allowing both the acute and late-phase respiratory physiological responses characteristic of allergic asthma to be studied in a single group of animals, and has been validated by direct comparison with the more invasive procedures previously mentioned (Hamelmann *et al.*, 1997).

Previous work in this laboratory with *Metarhizium anisopliae*, a fungal pesticide licensed for indoor control of cockroaches, has demonstrated that extracted fungal proteins administered to BALB/c mice are capable of increasing endpoints characteristic of allergic asthma, including eosinophilia and increased serum IgE levels (Ward *et al.*, 1998), as well as

airway hyperresponsiveness and inflammatory changes in lung pathology (Ward *et al.*, 2000). These endpoints are considered to be pathognomonic of human allergic asthma. In this study, we used this animal model to demonstrate a cause-effect relationship for exposure to *S. chartarum* and allergic asthma-type responses.

MATERIALS AND METHODS

Animals

Fifty-day-old female BALB/c mice (Charles River, Raleigh, NC) were group-housed in disposable polycarbonate cages with corncob bedding in an environmentally controlled, American Association for Accreditation of Laboratory Animal Care-accredited vivarium. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of NHEERL, U.S. EPA. Sentinel mice were monitored serologically and were found to be free of Sendai, mouse pneumonia, mouse hepatitis, other murine viruses, and mycoplasma. Mice also were monitored for, and found to be free of, ectoparasites and endoparasites. 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*. Individual animals were tracked by the subcutaneous implantation of a microchip (Labtrac, Avid Identification Systems, Inc., Norco, CA) one day after arrival. Mice were allowed to acclimate one week prior to the start of the experiment.

Fungal Antigen Preparation

Five *S. chartarum* isolates (58-07, 58-16, 58-17, 58-18, and 63-01, described in Vesper *et al.*, 1999) originally obtained from residences in Cleveland, OH (Etzel *et al.*, 1998) were grown on a nylon filter resting on plates of sheep's blood agar or cellulose agar at 23°C for one month and combined in approximately equal weight amounts. This mass was extracted by grinding with a sterile mortar and pestle for approximately 12 min in a total of 15 volumes (by weight) of Hanks' balanced salt solution (HBSS, Gibco BRL, Life Technologies Inc., Rockville, MD) + 0.05% Tween 80 (Fisher Scientific, Pittsburgh, PA). The resulting suspension was stirred overnight at 4°C, and then centrifuged at $12,500 \times g$ for 1 h at 4°C. The supernatant was decanted, adjusted to pH 6.0 with HCl, and filter sterilized with a 1.2 μm syringe filter followed by a 0.2 μm media filter. This sterile filtrate was concentrated with a stirred-cell concentrator (Amicon, Inc., Beverly, MA) using YM3 membranes (molecular weight ≥ 3000 Da retained). The crude antigen preparation (*S. chartarum* extract 1 [SCE-1]) was assayed for total protein concentration, as described below, and stored at -80°C until use. Endotoxin levels of the individual extracts were measured using a Limulus amebocyte lysate test kit (BioWhittaker, Walkersville, MD); levels ranged from 13.7-57.2 pg/ml (for comparison, USP standard for water for injection, 25 pg/ml).

Experimental Design

Three groups of 24 mice each were exposed by involuntary aspiration (IA) 4 times over a four-week period, as previously described in (Ward *et al.*, 1998), to 20, 10, or 5 μg SCE-1 in 50 μl total volume. Briefly, mice were anesthetized by halothane inhalation, and the dose of SCE-1 was deposited in the oropharynx. Animals inhaled the SCE-1 when their noses were gently occluded with a gloved fingertip. A group of control mice (vehicle control) was IA exposed concurrently 4 times over a four-week period to HBSS alone. There were no significant differences in the responses to the various doses; therefore, the data presented in Results is from the 10- μg dose group compared to the HBSS controls. An additional group of mice were IA exposed to 3 doses of HBSS and a final single dose of 20 μg SCE-1 in 50- μl total volume to serve as a nonspecific inflammation control. The responses of these animals were compared to results obtained from naïve animals. Additionally, a group of

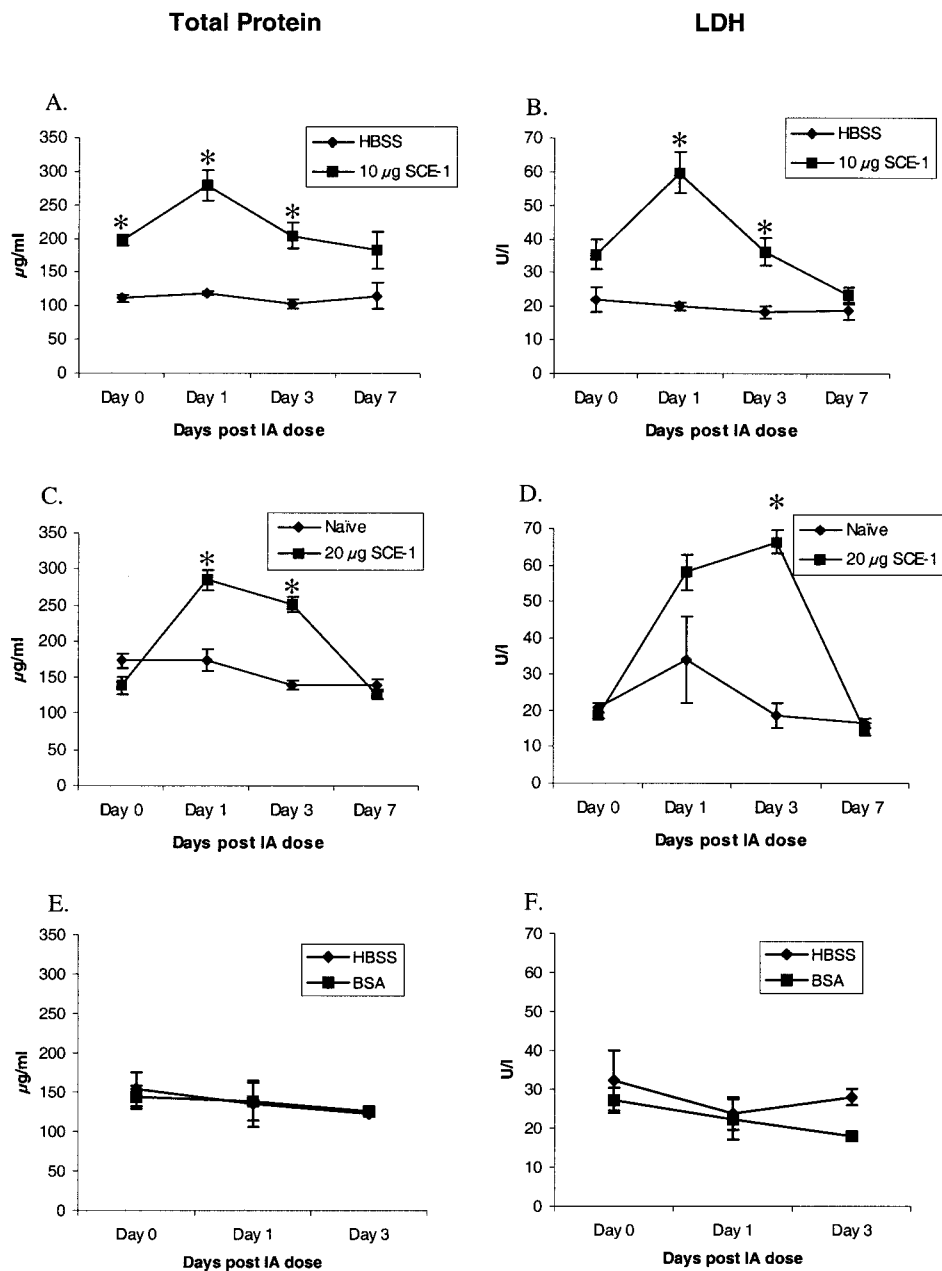


FIG. 1. (A) BALF total protein and (B) LDH levels in mice exposed to four 10- μ g doses of SCE-1 one week apart. (C) BALF total protein, (D) LDH levels in mice exposed to one 20- μ g dose of SCE-1, (E) BALF total protein and (F) LDH levels in mice exposed to four 10- μ g doses of BSA one week apart. *Significant difference from controls ($p < 0.05$). Error bars represent standard error of the mean; $n = 5-6$.

mice was exposed to 4 doses of 10 μ g bovine serum albumin (BSA) in 50- μ l total volume to serve as a nonallergenic protein control for both the amount of protein administered and for the route of exposure. For the experiments measuring airway responsiveness, mice were exposed 4 times to 10 μ g SCE-1, to a single 10 μ g dose of SCE-1, or to 4 doses of 10 μ g BSA, as described above. Antigen-specific immediate responses were measured after each dose, and responsiveness to methacholine was measured on Days 1 and 3 following the final dose, as described below.

Bronchoalveolar Lavage and Blood Collection

Blood and bronchoalveolar fluid (BALF) samples were collected as previously described (Ward *et al.*, 1998). Briefly, mice were anaesthetized 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

centrifugation, and the serum was stored at -80°C until analysis. The lungs were lavaged twice with 1 ml aliquots of HBSS, and the bronchoalveolar lavage fluid (BALF) aliquots were pooled and stored on ice. The BALF was centrifuged at $100 \times g$ for 15 min at 4°C to pellet the cells, the supernatant was decanted, and the cells were resuspended in 500 μ l of HBSS. Aliquots of BALF supernatant were assayed for total protein and lactate dehydrogenase (LDH) as described below, and the remainder was stored at -20°C for IgE and cytokine assays. Total BALF cell counts were performed using a hemacytometer and viability assessed by Trypan-blue dye exclusion. Cytology slides were made by centrifuging 100-150 μ l of resuspended cells onto glass slides at 200 rpm for 10 min using a Cytospin 2 centrifuge (Shandon Inc., Pittsburgh, PA). The BALF cells were stained with Wright-Giemsa (Fisher Scientific) on a Hema-Tek 2000 slide stainer (Miles, Inc., Elkhart, IN), and were differentially counted at 200 cells per slide (one slide per animal).

Total BALF Cell Number

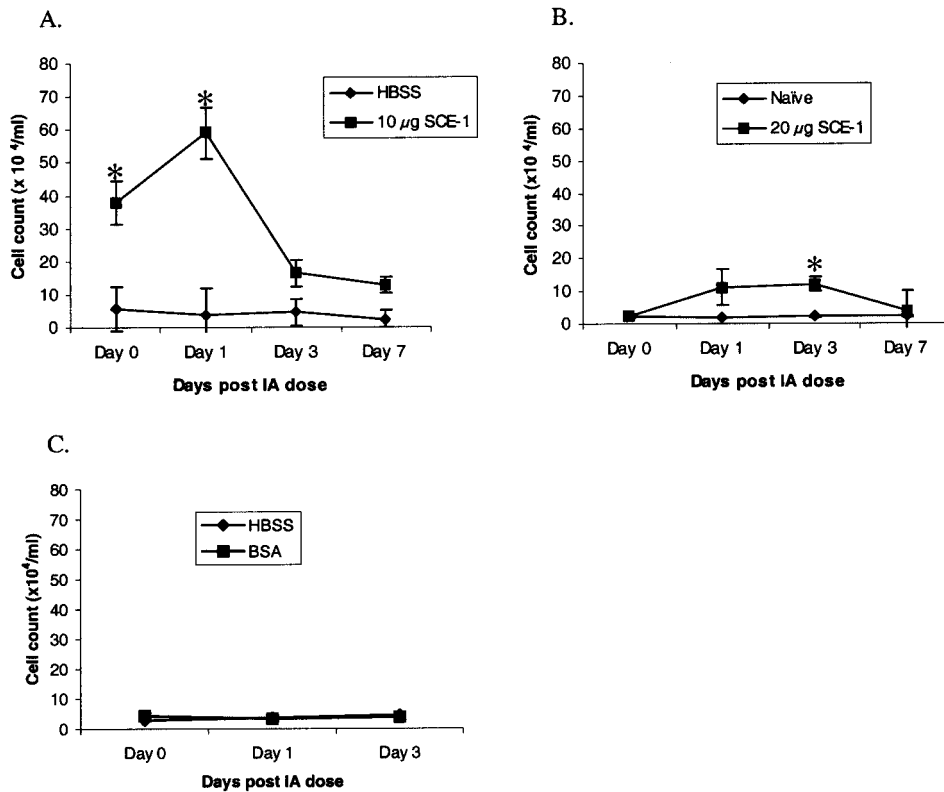


FIG. 2. (A) Total immune cell count in BALF from mice exposed to four 10- μ g doses of SCE-1 one week apart. (B) Total immune cell count in BALF from mice exposed to one 20- μ g dose of SCE-1. (C) Total immune cell count in BALF from mice exposed to four 10- μ g doses of BSA one week apart. *Significant difference from HBSS controls ($p < 0.05$). Error bars represent standard error of the mean; $n = 5-6$.

Total Protein and Lactate Dehydrogenase (LDH) Assays

BALF samples were assayed for total protein using Pierce Coomassie-Plus Protein Assay Reagent (Pierce, Rockford, IL). Concentrations were determined from a standard curve using BSA standards obtained from Sigma Chemical Co. (St. Louis, MO). Additionally, the BALF samples were assayed for LDH using a commercially prepared kit and controls (Sigma). The assay reagent proportions remained the same but volumes were reduced for use on a Cobas Fara II Centrifugal Spectrophotometer (Hoffman-LaRoche, Branchburg, NJ).

Total IgE, IgA, and IL-5 Enzyme-Linked Immunosorbent Assays (ELISAs)

Total IgE ELISA. All reagents and incubation periods were at room temperature to enhance assay precision and accuracy, and all volumes added were 100 μ l unless otherwise noted. Certified 96-well microtiter plates (Costar Corp., Cambridge, MA) were coated with rat anti-mouse IgE (PharMingen, San Diego, CA) at 3.0 μ g/ml in phosphate buffered saline (PBS), pH 7.3, sealed, and incubated overnight at 4°C. The plates were washed 3 times between each incubation with PBS + 0.5% Tween 20 (Fisher Scientific). To block nonspecific binding, 200 μ l of PBS + 1% BSA (Sigma) was added to plates. After 30 min, plates were washed as described above and either BALF samples (undiluted) or mouse serum (diluted 1:30 and 1:90 in blocking buffer), and purified mouse IgE standards (PharMingen, in 2-fold dilutions from 800 ng/ml to 0.78 ng/ml) were applied. Following a 1-h incubation and wash as described above, biotinylated rat anti-mouse IgE (PharMingen) at 2.5 μ g/ml in blocking buffer was added to all wells. After a 1-h incubation and 6 washes, streptavidin-horseradish peroxidase conjugate (Zymed Laboratories Inc., San Francisco, CA) at 0.16 μ g/ μ l was added and the plates incubated for 20 min. Following 6 washes, a 3,5'-5,5' tetramethylbenzidine substrate solution (TMB One-Step Substrate Solution, Dako Corporation, Carpinteria, CA) was added

and incubated for at least 20 min. Optical density was read on a SpectraMax 340PC Plate Reader (Molecular Devices Corp., Menlo Park, CA) at a wavelength of 650 nm. Softmax Pro® software (version 2.6.1, Molecular Devices Corp.) was used for data collection and conversion from optical density to protein concentrations.

Total IgA ELISA. The IgA ELISA was performed using the same protocol as described for the IgE ELISA. Briefly, microtiter plates were coated with anti-mouse IgA (PharMingen) at 3.0 μ g/ μ l in PBS (pH 7.3). BALF samples were diluted 1:100, and the IgA standard (purified mouse IgA, κ isotype, PharMingen) was added in 2-fold dilutions, providing a range of from 500 ng/ml to 0.49 ng/ml. The biotinylated anti-mouse IgA (PharMingen) was added at 2.5 μ g/ μ l. Streptavidin-horseradish peroxidase (Zymed Labs) and TMB substrate (Dako Corp.) were applied and optical density determined as described above. Softmax Pro® software was used for data collection and conversion from optical density to protein concentrations.

IL-5 ELISA. The IL-5 ELISA was performed as previously described (Ward *et al.*, 2000). Briefly, microtiter plates were coated with anti-mouse IL-5 (PharMingen) at 4.0 μ g/ μ l in 0.1 M sodium phosphate buffer (pH 9.0), sealed, and incubated overnight at 4°C. The plates were blocked with diluent buffer (PBS + 1.0% BSA + 0.05% Tween 20) for 1 h at room temperature, the IL-5 standard (PharMingen) was added in 2-fold dilutions (2000–1.95 pg/ml range), and the BALF samples were applied undiluted. The plates were sealed and incubated again overnight at 4°C. The biotinylated detection antibody (biotin-anti-mouse rIL-5, PharMingen) was diluted in diluent buffer, conjugated streptavidin-horseradish peroxidase (Zymed Labs) and TMB substrate (Dako Corp.) were applied, and optical density was determined as described above. Softmax Pro® software was used for data collection and conversion from optical density to protein concentrations.

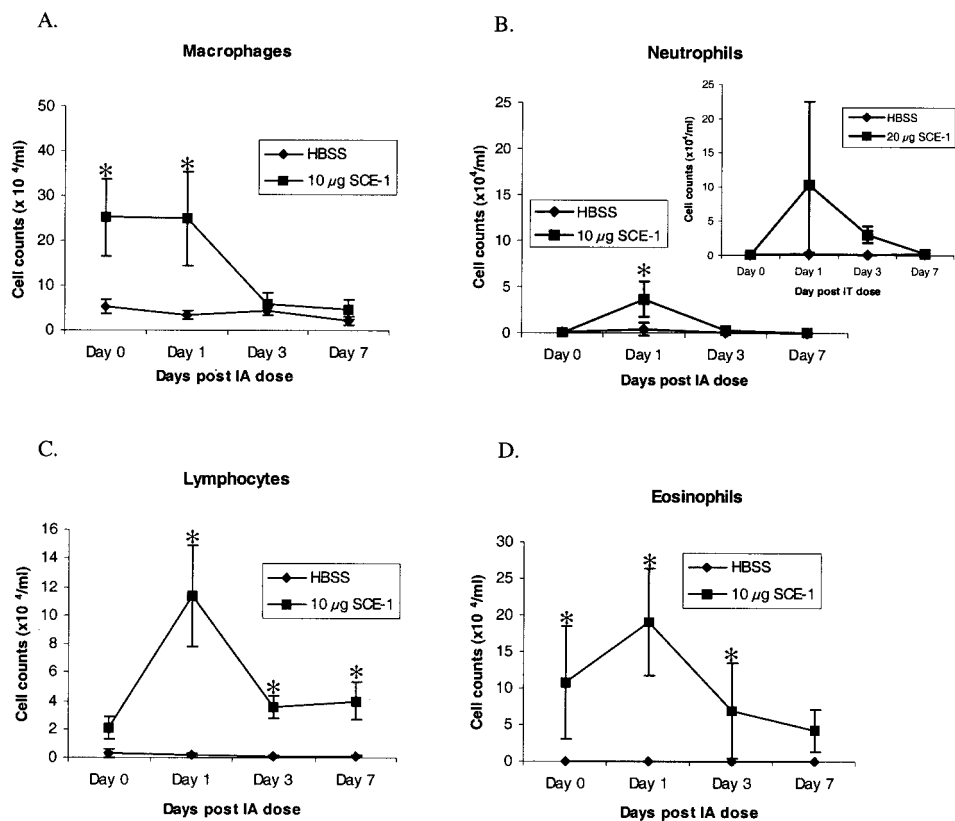


FIG. 3. Differential immune cell counts of BALF from mice exposed to four 10- μ g doses of SCE-1 one week apart; inset is from mice exposed to one 20- μ g dose of SCE-1. (A) Macrophages, (B) neutrophils, (C) lymphocytes, (D) eosinophils. *Significant difference from HBSS controls ($p < 0.05$). Error bars represent standard error of the mean; $n = 5-6$.

Histopathology

Following bronchoalveolar lavage, the lungs were inflated *in situ* with 10% buffered formalin acetate (Fisher Scientific, Fair Lawn, NJ), embedded in paraffin, and sectioned. One slide per mouse was prepared by sectioning the lung along the main stem bronchus and staining with hematoxylin-eosin solution prior to pathological analysis. The entire lung was examined at several magnifications and the relative degree of severity of inflammatory, degenerative, and proliferative changes was graded on a scale of 1 to 5 (1, minimal; 2, slight/mild; 3, moderate; 4, moderately severe; 5, severe/high). The pathology scores for lung sections were summarized as follows: incidence, the number of mice exhibiting certain pathology; severity, the numeric average of the pathology scores for a particular lesion per treatment group (protocol used by Experimental Pathology Laboratories, Inc., Research Triangle Park, NC). Diffuse alveolar macrophage accumulation consisted of activated macrophages present in alveolar air spaces in a large area of the lung parenchyma. Alveolitis consisted of focal thickening of alveolar septae, often with inflammatory cells in the affected alveolar spaces. Edema was diagnosed when there was a distinct distended area surrounding vessels that contained proteinaceous fluid.

Measurements of Airway Responsiveness

Antigen-specific immediate responses. Antigen-specific airway responsiveness was measured immediately after each exposure to SCE-1 (Days 28, 14, 7, 0) in unrestrained mice, using whole body plethysmography (Buxco Electronics, Troy, NY). In each plethysmograph, a pressure signal is generated from the pressure difference between the main chamber, containing the unrestrained mouse, and a reference chamber that cancels atmospheric disturbances. Signals were analyzed using BioSystem XA software (SFT3812, version 2.0.2.48, Buxco Electronics) to derive whole body flow parameters including respiratory rate, tidal volume, inspiratory time (Ti), expiratory time (Te), peak inspiratory flow (PIF), peak expiratory flow (PEF), and relaxation time (RT). These

parameters are used by the BioSystem XA software to calculate enhanced pause (PenH), a unitless parameter that strongly correlates with lung resistance (Hamelmann *et al.*, 1997). PenH reflects changes in pulmonary resistance during bronchoconstriction according to the following equation: $\text{PenH} = ((\text{Te} - \text{RT}) \div \text{RT}) \times (\text{PEF} \div \text{PIF})$. Baseline PenH measurements for each animal were recorded for 10 min and averaged. Animals were then IA exposed to SCE-1, and placed back in the chambers within 5 min of dosing. PenH readings were then monitored and averaged over a 1-hour post-instillation period.

Airway responsiveness to methacholine aerosol. Airway responsiveness to methacholine (MCh) aerosol was determined by measurement of time-integrated changes in PenH in response to MCh on Days 1 and 3 after the final exposure to SCE-1. After measurement of baseline PenH for 5 min, either saline or MCh in increasing concentrations (4, 8, 16, and 32 mg/ml) was nebulized and delivered through an inlet of the chamber for 1 min, and measurements of PenH were made for 4, 5, 6, 7 and 11 min, respectively, after each dose. After subtracting baseline values, time-integrated changes in PenH were calculated and expressed as areas under the curve (AUC) for each concentration of MCh (PenH units $\times$ s). Immediate responses and methacholine responsiveness were analyzed across treatment groups and within individual mice on different days of the protocol. The interaction between the treatment and day effects was also examined.

Statistics

The data were analyzed using analysis of variance. Pairwise comparisons were performed as subtests of the overall model. In cases of comparing a control group to other groups, Dunnett's test was used. Significance was attributed to an effect or difference if the probability was less than 0.05. Adjustments in significance levels of multiple comparisons were made, using a modified Bonferroni correction.

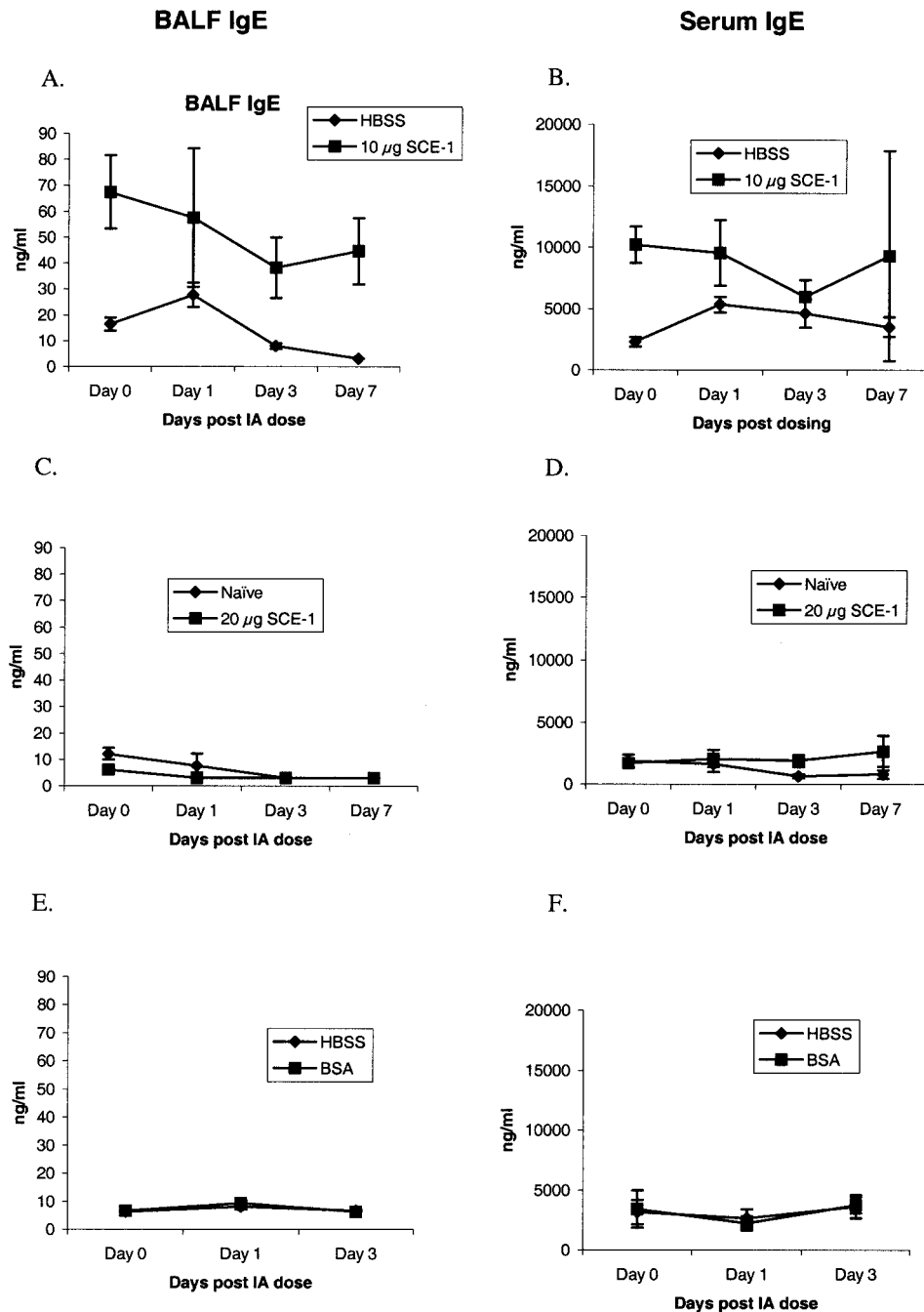


FIG. 4. (A) BALF and (B) serum IgE levels in mice exposed to four 10- μ g doses of SCE-1 one week apart. There was a significant treatment effect ($p < 0.05$) in both cases. (C) BALF and (D) serum IgE levels in mice exposed to one 20- μ g dose of SCE-1. (E) BALF and (F) serum IgE levels in mice exposed to four 10- μ g doses of BSA 1 week apart. Error bars represent standard error of the mean; $n = 5-6$. Limits of detection: (A) 12.5 ng/ml, (B) 6.25 ng/ml, (C) 3.125 ng/ml, (D) 3.125 ng/ml, (E) 6.25 ng/ml, and (F) 6.25 ng/ml.

RESULTS

Total protein and LDH. Total protein and LDH levels in BALF were measured as nonspecific indices of edema and cellular damage, respectively. Mice treated with 4 doses of SCE-1 showed a significant increase in total protein levels (Fig. 1A) at Day (D) 0, 1, and 3 after the last IA exposure, compared to HBSS-treated controls. Levels were still elevated, but not significantly, at Day 7. LDH levels (Fig. 1B) in SCE-1-treated mice were elevated at Day 0, and significantly increased at Day 1 and

Day 3 after the last IA exposure, compared to HBSS-treated controls; LDH levels returned to control levels by Day 7. The total protein and LDH profiles in mice exposed to a single 20 μ g dose of SCE-1 was similar to those of mice exposed 4 times (Figs. 1C and 1D). In contrast, mice exposed to 4 doses of BSA showed no changes in either total protein (Fig. 1E) or LDH levels (Fig. 1F) compared to HBSS-treated controls.

BALF total and differential cell counts. As shown in Figure 2A, mice treated with 4 doses of SCE-1 had significantly

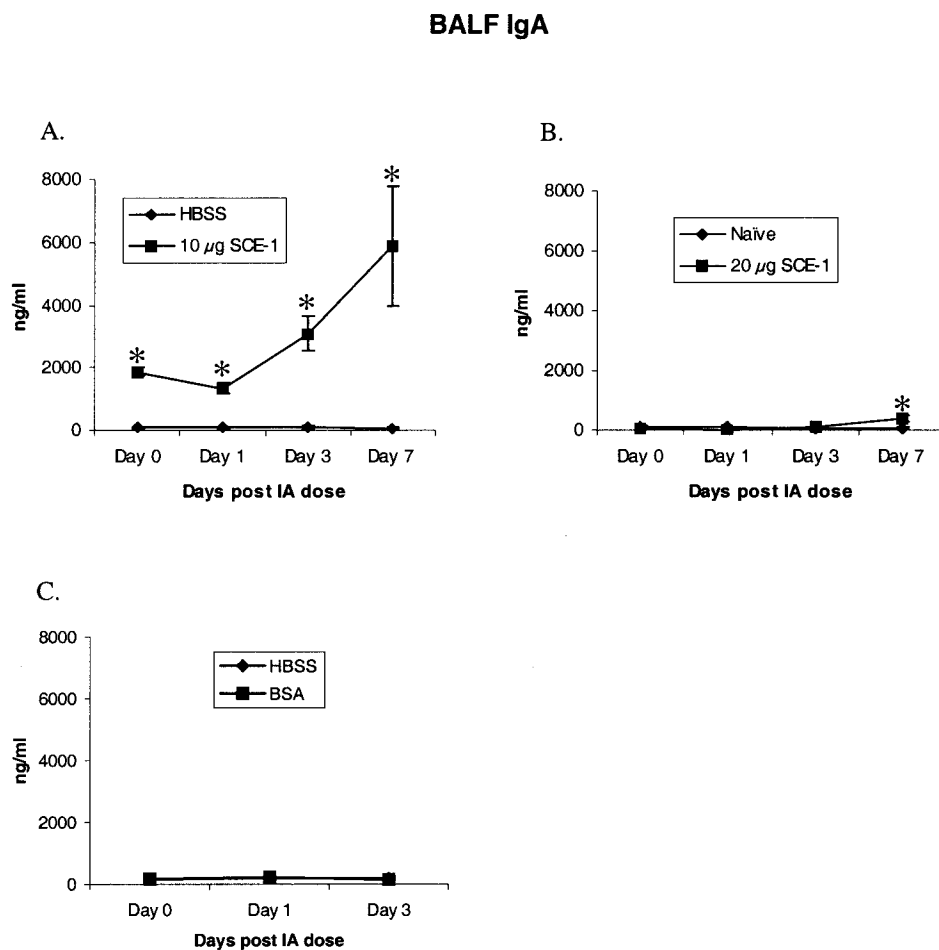


FIG. 5. (A) BALF IgA levels in mice exposed to four 10- μ g doses of SCE-1 one week apart. (B) BALF IgA levels in mice exposed to one 20- μ g dose of SCE-1. (C) BALF IgA levels in mice exposed to four 10- μ g doses of BSA one week apart. *Significant difference from controls ($p < 0.05$). Error bars represent standard error of the mean; $n = 5-6$. Limits of detection: (A) 1.00 ng/ml, (B) 0.781 ng/ml, and (C) 0.781 ng/ml.

increased total BALF cell numbers at Days 0 and 1 after the last IA exposure compared to HBSS controls. Total cell numbers remained elevated over control values at Days 3 and 7, but not at a statistically significant level. Mice receiving a single 20 μ g dose of SCE-1 showed smaller increases in cell numbers at Day 1 and Day 3 that resolved to naïve levels by Day 7 (Fig. 2B). Mice treated with 4 doses of BSA did not have increased BALF cell numbers when compared to HBSS controls (Fig. 2C). Differential counts revealed the predominant cells present in SCE-1 BALF at Day 0 were macrophages (Fig. 3A). SCE-1 BALF showed a significant influx of neutrophils (Fig. 3B) noted at Day 1 that resolved to control numbers by Day 3. SCE-1-treated mice also had significantly increased numbers of lymphocytes (Fig. 3C) in BALF at Day 1, Day 3, and Day 7, and significantly increased numbers of eosinophils (Fig. 3D) at Day 0, Day 1, and Day 3. BALF from HBSS-treated mice had very small numbers of neutrophils and lymphocytes, and there were no eosinophils present at any time following the final IA exposure. Differential cell counts of BALF from mice exposed to a single 20- μ g dose of SCE-1 (Fig. 3B, inset) showed a large neutrophil influx at Day 1 that resolved to naïve levels by Day 7. These animals also demonstrated a smaller but statistically

significant influx of lymphocytes at Day 3 that remained elevated over naïve levels at Day 7; however, no eosinophils were observed at any time point (data not shown). Mice treated with 4 doses of BSA exhibited differential BALF cell counts that were not different from HBSS controls (data not shown).

IgE ELISA. Elevated IgE levels are considered to be indicative of an allergic response. Mice treated 4 times with SCE-1 demonstrated increased levels of total IgE in BALF (Fig. 4A) and serum (Fig. 4B) at all time points following the final IA exposure, compared to HBSS-treated controls. Statistical analysis showed that there was a significant treatment effect ($p < 0.05$); however, there was not a significant day effect and therefore not a significant treatment $\times$ day effect. In contrast, BALF IgE levels in mice receiving a single 20- μ g dose of SCE-1 (Fig. 4C) were at the limit of detection (3.13 ng/ml), while serum IgE levels were not significantly different from naïve animals (Fig. 4D). Similarly, animals receiving 4 doses of BSA had IgE levels comparable to HBSS-treated controls in both BALF and serum (Figs. 4E and 4F).

IgA ELISA. IgA is the predominant antibody isotype found in mucosal secretions. As shown in Figure 5A, levels of BALF

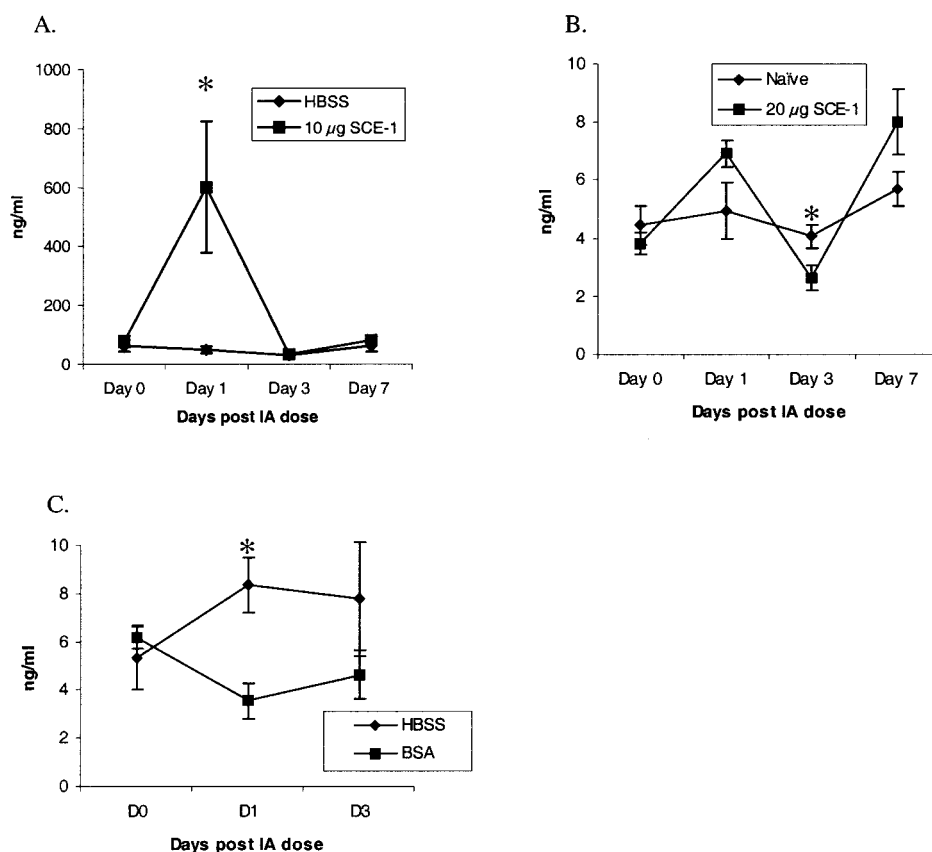


FIG. 6. (A) BALF IL-5 levels in mice exposed to four 10- μ g doses of SCE-1 one week apart. (B) BALF IL-5 levels in mice exposed to one 20- μ g dose of SCE-1. (C) BALF IL-5 levels in mice exposed to four 10- μ g doses of BSA one week apart. *Significant difference from HBSS controls ($p < 0.05$). Error bars represent standard error of the mean; $n = 5-6$. Limits of detection: (A) 31.25 ng/ml, (B) 3.91 ng/ml, (C) 1.95 ng/ml.

IgA were significantly elevated in SCE-1-treated mice compared to controls, throughout the time course. Mice receiving a single 20- μ g dose of SCE-1 had IgA levels comparable to naïve animals at Day 0, Day 1, and Day 3, with levels rising slightly at Day 7 (Fig. 5B). Mice treated with 4 doses of BSA did not have elevated BALF IgA levels compared to HBSS controls (Fig. 5C).

IL-5 ELISA. IL-5 is the predominant cytokine involved in the production, activation, and localization of eosinophils (reviewed in Sanderson, 1992). As shown in Figure 6A, BALF IL-5 levels in SCE-1-treated mice demonstrated a large significant increase at Day 1 compared to controls, returning to control levels at Day 3. Mice receiving a single 20- μ g dose of SCE-1 had IL-5 levels comparable to naïve animals at all time points (Fig. 6B). Mice treated with 4 doses of BSA did not have elevated BALF IL-5 levels compared to HBSS controls (Fig. 6C).

Pathology. The pathology data correlated well with the biochemical indices measured. As shown in Table 1, mice exposed to SCE-1 exhibited a variety of pulmonary pathological diagnoses that were primarily inflammatory in nature. Hypertrophy of the bronchiolar epithelium was characterized by the presence of a "slightly eosinophilic foamy cytoplasm," with only hypertrophy and not hyperplasia noted. The mixed cellular response was characterized by predominantly poly-

morphonuclear cells at Day 0 and Day 1, the majority of which were eosinophils. By Day 3, the response was composed of more mononuclear cells, consisting mainly of lymphocytes and mononuclear phagocytic cells. The chronic-active alveolar inflammation was noted to consist of a mixture of polymorphonuclear cells, eosinophils and neutrophils, mononuclear inflammatory cells, and a small number of multinucleated giant cells. Edema was also noted around the larger pulmonary vessels, particularly in animals exhibiting more severe inflammatory responses at Day 1 and Day 3. HBSS-treated controls

TABLE 1
Summary of the Incidence and Severity of Lung Pathology following Exposure to 4 Doses of 10 μ g SCE-1

Pathology (tissue)	Incidence (severity)			
	Day 0	Day 1	Day 3	Day 7
Hypertrophy, epithelium, bronchiole	5/5 (2.0)	5/5 (2.0)	5/5 (2.0)	5/5 (1.6)
Infiltration, mixed cell, peribronchial	5/5 (1.0)	5/5 (2.0)	5/5 (2.0)	5/5 (1.8)
Infiltration, mixed cell, perivascular	5/5 (2.0)	5/5 (2.8)	5/5 (2.6)	5/5 (2.8)
Inflammation, chronic-active, alveolus	5/5 (1.0)	5/5 (2.0)	5/5 (1.4)	5/5 (1.4)

Note. Incidence is the number of animals with lesions/total number of animals. Severity is the average score for each group (1, minimal; 2, slight/mild; 3, moderate; 4, moderately severe; 5, severe/high).

TABLE 2
Summary of Baseline PenH Measurements
for HBSS, HBSS/SCE-1, and SCE-1

Aspiration no.	Treatment		
	HBSS	HBSS/SCE-1	SCE-1
1	0.79 ± 0.014	0.81 ± 0.017	0.85 ± 0.006
2	0.80 ± 0.013	0.81 ± 0.017	0.86 ± 0.006
3	0.81 ± 0.013	0.82 ± 0.016	0.98 ± 0.007
4	0.81 ± 0.013	0.81 ± 0.016	1.10 ± 0.007

exhibited only a mild alveolar infiltration of macrophages (data not shown).

Antigen-specific immediate responses. Baseline measurements of enhanced pause (PenH) were performed for 10 min prior to each of the aspiration exposures, as shown in Table 2. Mice given 10 μ g doses of SCE-1 demonstrated a significant increase in baseline PenH at the 3rd and 4th exposures, respectively. Treatment with 4 doses of HBSS or 3 doses of HBSS followed by a 10- μ g dose of SCE-1 did not cause increases in baseline PenH at any exposure. Immediate respiratory responses were measured for 1 h following each aspiration exposure. Animals exposed to 10 μ g doses of SCE-1 exhibited a small (approximately 10%) but statistically significant increase in PenH over baseline after the 2nd exposure. This increase continued with each subsequent exposure, rising to 470% over baseline after the 3rd exposure, and 560% above baseline after the 4th exposure (Fig. 7A). Treatment with 4 doses of HBSS or 3 doses of HBSS followed by a 10- μ g dose of SCE-1 did not cause increases in immediate responses over baseline at any exposure. Further, exposure to 4 doses of BSA did not alter baseline PenH values (data not shown), nor cause increases in immediate responses over baseline PenH after any exposure (Fig. 7B).

Airway responsiveness to methacholine aerosol. Individuals suffering from allergic asthma demonstrate exaggerated bronchial airway hyperreactivity following exposure to the nonspecific cholinergic agonist methacholine (MCh). Therefore, airway hyperresponsiveness to increasing concentrations of nebulized MCh was assessed in all groups on days 1 and 3 following the 4th aspiration exposure. As shown in Figure 8A, animals exposed to 4 doses of SCE-1 exhibit a significant increase in PenH at 8, 16, and 32 mg/ml of inhaled MCh on Day 1 following the final IA exposure, compared to both HBSS controls and mice receiving 3 doses of HBSS followed by a 10- μ g dose of SCE-1. This hyperresponsiveness persisted to day 3, when SCE-1-treated mice showed an increased PenH following a 32-mg/ml MCh aerosol challenge (Fig. 8B). Mice exposed to 4 doses of HBSS or 3 doses of HBSS followed by a 10- μ g dose of SCE-1 were not statistically different from each other at any MCh concentration on either Day 1 or 3. Exposure to 4 doses of BSA did not alter PenH values com-

pared to HBSS treatment at any MCh concentration (Fig. 8C) on Day 1 after the 4th IA exposure.

DISCUSSION

Our results indicate that mice sensitized and challenged by respiratory exposure to SCE-1 developed both nonspecific inflammatory responses as well as many of the responses associated with respiratory allergy and asthma. Specifically, mice receiving 4 doses of SCE-1 had increased BALF and serum IgE levels, significant influxes of lymphocytes and eosinophils, and increased levels of the Th2 cytokine IL-5. In contrast, animals exposed to only one dose of SCE-1 demonstrated nonspecific inflammatory responses, including elevated BALF protein and LDH levels and neutrophilia, but did not have elevations in levels of IgE, IL-5, or eosinophilia in BALF. It should be noted these animals received twice the SCE-1 total protein (20 μ g) in a single dose compared to animals that received 4 doses of 10 μ g per dose; however the magnitudes of the inflammatory measures were approximately equivalent, suggesting a maximal nonspecific response to the fungal extract at both doses. Additionally, it is also possible that these nonspecific inflammatory reactions may facilitate sensitization

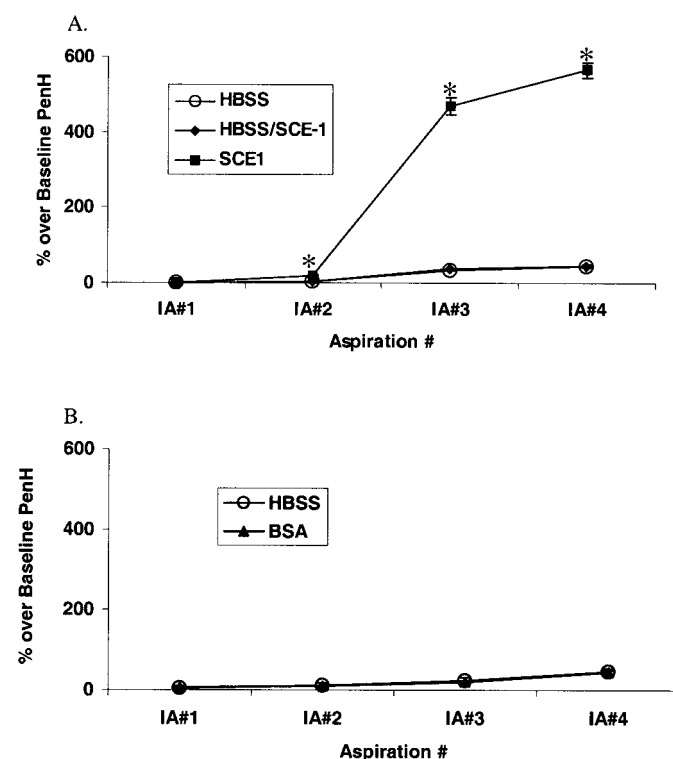


FIG. 7. (A) Immediate respiratory physiological responses in mice exposed to 4 doses of HBSS, 3 doses of HBSS plus 1 dose of SCE-1, or 4 doses of SCE-1; $n = 10$ –12. (B) Immediate respiratory physiological responses in mice exposed to 4 doses of HBSS or 4 doses of BSA; $n = 3$. *Significant difference from HBSS controls ($p < 0.05$). Error bars represent standard error of the mean.

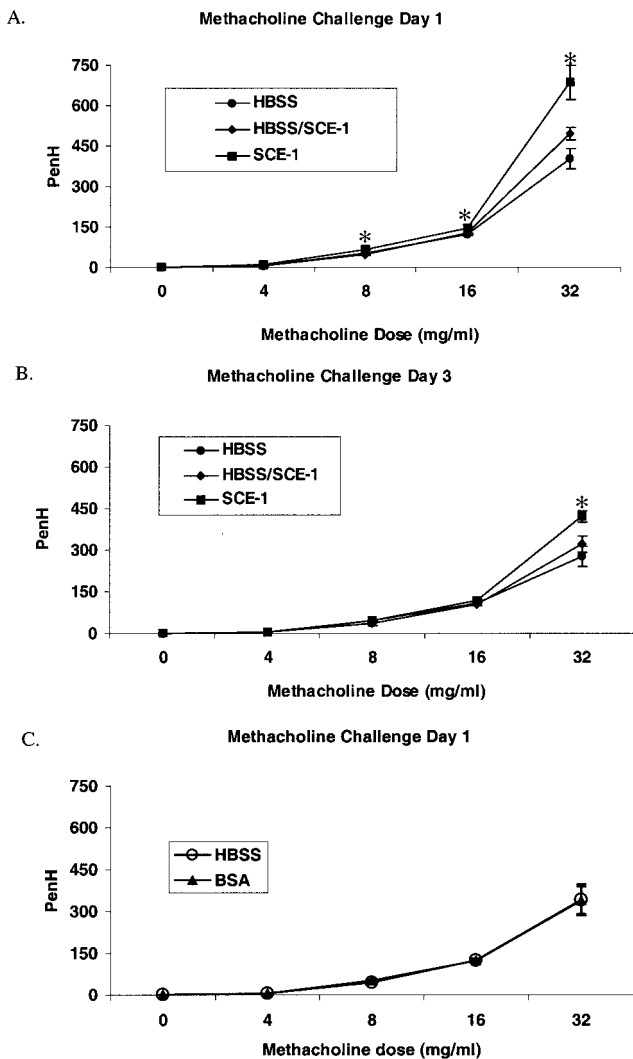


FIG. 8. Respiratory hyperreactivity responses to doubling doses of methacholine in mice previously treated with 4 doses of HBSS, 3 doses of HBSS plus 1 dose of SCE-1, or 4 doses of SCE-1. (A) Day 1 after final IA exposure; $n = 10-12$. (B) Day 3 after final IA exposure; $n = 5-6$. (C) Respiratory hyperreactivity responses to doubling doses of methacholine in mice previously treated with 4 doses of HBSS or 4 doses of BSA on Day 1 after final IA exposure; $n = 3$. *Significant difference from HBSS controls ($p < 0.05$). Error bars represent standard error of the mean.

and the specific allergic responses by mobilizing pulmonary immune cells and/or increasing lung permeability.

It is also interesting to note the dramatic increase in total BALF IgA levels we observed in mice receiving multiple doses of SCE-1. Serum IgA levels are increased in active cases of farmer's lung disease (Kaukonen *et al.*, 1993), as well as in induced sputum from asthmatics (Nahm *et al.*, 1998). While both IgG and IgA are capable of triggering eosinophil degranulation, secretory IgA is the most potent isotype (Abu-Ghazaleh *et al.*, 1989), as well as the principal antibody found on mucosal surfaces. Animals that received one dose of SCE-1 showed a small but significant increase in IgA levels at day 7,

as one would expect in the development of a humoral response, whereas BSA-treated mice did not exhibit an IgA response. We propose IgA could be another indicator of an allergic response, when accompanied by other accepted parameters, such as eosinophilia and increased IgE levels.

Airway narrowing following exposure to an inhaled allergen is the primary physiological response in allergic asthma, and airway hyperresponsiveness to a nonspecific stimulus such as methacholine is a diagnostic tool used in patients with suspected asthma. We used a barometric whole-body plethysmography system to measure both immediate airway bronchoconstriction and airway hyperresponsiveness in unanesthetized, unrestrained mice (Hamelmann *et al.*, 1997). This method allowed repeated measurements to be performed, enabling us to determine both the acute immediate responses and longer-lasting airway hyperresponsiveness following allergen exposure in the same animals. Further, this system allowed us to administer the bronchoconstricting agent methacholine as an inhaled nebulized mist, rather than by injection. This avoids potential interactions of other organ systems that would have been exposed to higher concentrations of a nonspecific cholinergic agonist, and uses the same route of administration and concentrations as those used in clinical settings.

In the present study, mice previously sensitized to SCE-1 had both increased immediate bronchoconstriction upon SCE-1 challenge and increased airway hyperresponsiveness to the nonspecific methacholine challenge. We observed a small statistically significant increase in bronchoconstriction after the 2nd exposure. This indicates the first dose was sufficient to cause sensitization, and a single challenge was sufficient to evoke physiological responses correlating with the allergic and inflammatory biochemical and pathological alterations observed. The subsequent 3rd and 4th exposures appeared to drive the responses toward a peak with no evidence of a decline, indicative of tolerance, after the last dose. In contrast, animals exposed to a single dose of SCE-1 did not demonstrate immediate bronchoconstriction nor increased airway hyperresponsiveness to a subsequent nonspecific challenge, despite the presence of biochemical and pathological indices of pulmonary inflammation. This argues that the acute and methacholine-induced respiratory physiological responses we observed are allergen-induced, and not an artifact of a nonspecific inflammatory response. Further, the biochemical and pathological responses, particularly increases in IgE and the presence of eosinophils, were predictive of physiological changes in exposed mice.

Another concern of studies utilizing the respiratory route of sensitization and challenge was that any protein introduced into the lungs in this fashion could cause inflammation and/or allergic responses similar to the ones we observed. BSA was selected as a candidate for a negative control protein, based on the works reported by Dearman *et al.* (2000) and Hilton *et al.* (1997). Their work, while utilizing different routes of exposure and greater amounts of protein, suggested that BSA either would not provoke an allergic response, or would provoke a

weak response. BSA did not cause increases in any of the inflammatory or allergic parameters we measured. Therefore, our findings indicate that BSA is both nonallergenic and non-inflammatory when administered by aspiration at the dose level we used (10 μ g), even when administered multiple times, and it can serve as a negative control for this experimental protocol in future studies.

There was a possibility that the amino acid sequences of BSA and mouse serum albumin were similar enough to be viewed as "self" by the immune system, and therefore, incapable of provoking an immune response. A BLAST comparison of BSA (accession no. AAA51411) and mouse serum albumin (accession no. CAD29888) revealed an amino acid identity of only 68% (416 of 608), indicating substantial sequence differences between these molecules. Also, both Hilton *et al.* (1997) and Dearman *et al.* (2000) reported that intranasal exposure to BSA provoked a vigorous IgG response, indicating BSA was recognized as a foreign protein by the mouse immune system. Thus, BSA appears to be antigenic, but not allergenic, when administered without adjuvant to BALB/c mice.

We have shown responses in mice exposed to *S. chartarum* extract that are consistent with human allergic airway disease. Therefore, we conclude there are proteins in this aqueous extract capable of inducing allergy in a susceptible population. Future studies to isolate and characterize the specific allergens are planned, as are studies to determine if nonallergenic components of the extract function as an adjuvant. The BALB/c mouse model provides the means to address these issues and should also be useful in assessing the potential allergic responses and relative potencies of other fungal organisms found to contaminate the indoor environment.

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REFERENCES

- Abu-Ghazaleh, R. I., Fujisawa, T., Mestecky, J., Kyle, R. A., and Gleich, G. J. (1989). IgA-induced eosinophil degranulation. *J. Immunol.* **142**, 2393–2400.
- CDC (2001). Self-reported asthma prevalence among adults—United States, 2000. *MMWR Morb. Mortal. Wkly. Rep.* **50**, 682–686.
- Cooley, J. D., Wong, W. C., Jumper, C. A., and Straus, D. C. (1998). Correlation between the prevalence of certain fungi and sick-building syndrome. *Occup. Environ. Med.* **55**, 579–584.
- Croft, W. A., Jarvis, B. B., and Yatawara, C. S. (1986). Airborne outbreak of trichothecene toxicosis. *Atmospher. Environ.* **20**, 549–552.
- Dearman, R. J., Caddick, H., Basketter, D. A., and Kimber, I. (2000). Divergent antibody isotype responses induced in mice by systemic exposure to proteins: A comparison of ovalbumin with bovine serum albumin. *Food Chem. Toxicol.* **38**, 351–360.
- Elidemir, O., Colasurdo, G. N., Rossmann, S. N., and Fan, L. L. (1999). Isolation of *Stachybotrys* from the lung of a child with pulmonary hemosiderosis. *Pediatrics* **104**, 964–966.
- Etzel, R. A., Montana, E., Sorenson, W. G., Kullman, G. J., Allan, T. M., Dearborn, D. G., Olson, D. R., Jarvis, B. B., and Miller, J. D. (1998). Acute pulmonary hemorrhage in infants associated with exposure to *Stachybotrys atra* and other fungi. *Arch. Pediatr. Adolesc. Med.* **152**, 757–762.
- Hamelmann, E., Schwarze, J., Takeda, K., Oshiba, A., Larsen, G. L., Irvin, C. G., and Gelfand, E. W. (1997). Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* **156**, 766–775.
- Hilton, J., Dearman, R. J., Sattar, N., Basketter, D. A., and Kimber, I. (1997). Characteristics of antibody responses induced in mice by protein allergens. *Food Chem. Toxicol.* **35**, 1209–1218.
- Hodgson, M. J., Morey, P., Leung, W. Y., Morrow, L., Miller, D., Jarvis, B. B., Robbins, H., Halsey, J. F., and Storey, E. (1998). Building-associated pulmonary disease from exposure to *Stachybotrys chartarum* and *Aspergillus versicolor*. *J. Occup. Environ. Med.* **40**, 241–249.
- Holt, P. G., Macaubas, C., Stumbles, P. A., and Sly, P. D. (1999). The role of allergy in the development of asthma. *Nature* **402**, B12–17.
- Jarvis, B. B., Salemme, J., and Morais, A. (1995). *Stachybotrys* toxins 1. *Nat. Toxins* **3**, 10–16.
- Jarvis, B. B., Sorenson, W. G., Hintikka, E. L., Nikulin, M., Zhou, Y., Jiang, J., Wang, S., Hinkley, S., Etzel, R. A., and Dearborn, D. (1998). Study of toxin production by isolates of *Stachybotrys chartarum* and *Memnoniella echinata* isolated during a study of pulmonary hemosiderosis in infants. *Appl. Environ. Microbiol.* **64**, 3620–3625.
- Kaukonen, K., Savolainen, J., Viander, M., Kotimaa, M., and Terho, E. O. (1993). IgG and IgA subclass antibodies against *Aspergillus umbrosus* in farmer's lung disease. *Clin. Exp. Allergy* **23**, 851–856.
- Kobayashi, T., Miura, T., Haba, T., Sato, M., Serizawa, I., Nagai, H., and Ishizaka, K. (2000). An essential role of mast cells in the development of airway hyperresponsiveness in a murine asthma model. *J. Immunol.* **164**, 3855–3861.
- Mahmoudi, M., and Gershwin, M. E. (2000). Sick building syndrome. III. *Stachybotrys chartarum*. *J. Asthma* **37**, 191–198.
- Martin, T. R., Gerard, N. P., Galli, S. J., and Drazen, J. M. (1988). Pulmonary responses to bronchoconstrictor agonists in the mouse. *J. Appl. Physiol.* **64**, 2318–2323.
- Montana, E., Etzel, R. A., Allan, T., Horgan, T. E., and Dearborn, D. G. (1997). Environmental risk factors associated with pediatric idiopathic pulmonary hemorrhage and hemosiderosis in a Cleveland community. *Pediatrics* **99**, E5, 1–8.
- Nahm, D. H., Kim, H. Y., and Park, H. S. (1998). Elevation of specific immunoglobulin A antibodies to both allergen and bacterial antigen in induced sputum from asthmatics. *Eur. Respir. J.* **12**, 540–545.
- Novotny, W. E., and Dixit, A. (2000). Pulmonary hemorrhage in an infant following 2 weeks of fungal exposure. *Arch. Pediatr. Adolesc. Med.* **154**, 271–275.
- Pieckova, E., and Jesenska, Z. (1999). Microscopic fungi in dwellings and their health implications in humans. *Ann. Agric. Environ. Med.* **6**, 1–11.
- Renz, H., Smith, H. R., Henson, J. E., Ray, B. S., Irvin, C. G., and Gelfand, E. W. (1992). Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. *J. Allergy Clin. Immunol.* **89**, 1127–1138.
- Sanderson, C. J. (1992). Interleukin-5, eosinophils, and disease. *Blood* **79**, 3101–3109.
- Sorenson, W. G., Frazer, D. G., Jarvis, B. B., Simpson, J., and Robinson, V. A. (1987). Trichothecene mycotoxins in aerosolized conidia of *Stachybotrys atra*. *Appl. Environ. Microbiol.* **53**, 1370–1375.

- Travis, E. L., Brightwell, D., Aiken, M., and Boyd, M. R. (1982). Whole body plethysmography as a noninvasive assay of toxic lung injury in mice: Studies with the pulmonary alkylating agent and cytotoxin, 4-ipomeanol. *Toxicol. Appl. Pharmacol.* **66**, 193–200.
- Tripi, P. A., Modlin, S., Sorenson, W. G., and Dearborn, D. G. (2000). Acute pulmonary haemorrhage in an infant during induction of general anaesthesia. *Paediatr. Anaesth.* **10**, 92–94.
- Vesper, S. J., Dearborn, D. G., Yike, I., Sorenson, W. G., and Haugland, R. A. (1999). Hemolysis, toxicity, and randomly amplified polymorphic DNA analysis of *Stachybotrys chartarum* strains. *Appl. Environ. Microbiol.* **65**, 3175–3181.
- Vesper, S. J., Magnuson, M. L., Dearborn, D. G., Yike, I., and Haugland, R. A. (2001). Initial characterization of the hemolysin stachylysin from *Stachybotrys chartarum*. *Infect. Immun.* **69**, 912–916.
- Ward, M. D., Madison, S. L., Sailstad, D. M., Gavett, S. H., and Selgrade, M. K. (2000). Allergen-triggered airway hyperresponsiveness and lung pathology in mice sensitized with the biopesticide *Metarhizium anisopliae*. *Toxicology* **143**, 141–154.
- Ward, M. D., Sailstad, D. M., and Selgrade, M. K. (1998). Allergic responses to the biopesticide *Metarhizium anisopliae* in Balb/c mice. *Toxicol. Sci.* **45**, 195–203.
- Weiss, K. B., and Sullivan, S. D. (2001). The health economics of asthma and rhinitis: I. Assessing the economic impact. *J. Allergy Clin. Immunol.* **107**, 3–8.