

Characterization of Exposure to Low Levels of Viable *Penicillium chrysogenum* Conidia and Allergic Sensitization Induced by a Protease Allergen Extract from Viable *P. chrysogenum* Conidia in Mice

Christopher J. Schwab^a J. Danny Cooley^a Trevor Brasel^a
Cynthia A. Jumper^b Suzanne C. Graham^c David C. Straus^a

Departments of ^aMicrobiology and Immunology, ^bInternal Medicine and ^cPathology, Texas Tech University Health Sciences Center, Lubbock, Tex., USA

Key Words

Penicillium chrysogenum · Mold · Conidia · Sick building syndrome · Murine model · Eosinophils · IgE · IgG1 · Allergy

Abstract

Background: Previous evidence by our laboratory has shown that mice inoculated with viable *Penicillium chrysogenum* conidia or spores at levels comparable to those found in contaminated buildings induced spore antigen-specific allergic responses. We proposed that mice exposed to low levels of viable *P. chrysogenum* conidia would not develop allergic symptoms. We also hypothesized that the symptoms induced by high numbers of conidia were the result of sensitization to allergens released by the conidia. **Methods:** C57BL/6 and BALB/c mice were exposed to 1×10^2 viable *P. chrysogenum* conidia by intranasal instillation weekly for a period of 11 weeks. C57BL/6 mice were also sensitized to a viable *P. chrysogenum* conidia protease extract by intraperitoneal injections for a period of 6 weeks followed by intranasal challenge with protease extract, viable, or nonviable *P. chrysogenum* conidia for 2 weeks. **Results:** C57BL/6 mice

inoculated with low numbers of conidia developed no significant lung inflammation or increased serum immunoglobulins. Mice sensitized to the protease extract and challenged with both protease extract and viable conidia produced significant increases in serum IgE and IgG1. Mice sensitized to and challenged with the protease extract developed significant eosinophilia and mucus hyperproduction as determined by bronchoalveolar lavage and histopathological examination of lung tissue. **Conclusions:** Mice did not develop allergic symptoms in response to challenge with low levels of *P. chrysogenum* conidia. Protease allergens from viable conidia induced specific allergic responses in mice, indicating the importance of *P. chrysogenum* conidia in allergic sensitization to the organism.

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Introduction

Sick building syndrome (SBS), a commonly used term describing specific health complaints by people that work or live in buildings with indoor air quality (IAQ) problems, includes symptoms such as allergic rhinitis, head-

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Correspondence to: Dr. David C. Straus, PhD
Department of Microbiology and Immunology, TTUHSC
3601 4th Street
Lubbock, TX 79430 (USA)
Tel. +1 806 743 2523, Fax +1 806 743 2334, E-Mail david.straus@ttmc.ttuhsu.edu

aches, flu-like complaints, watering of eyes, and difficulty in breathing [1, 2]. Recent studies have implicated fungi and their products in IAQ problems [3, 4] as well as allergies in indoor environments [5, 6]. Recent evidence by our laboratory has shown a correlation between the presence of specific fungal species and SBS symptoms as well as IAQ complaints [7, 8]. Conidia from the organism *Penicillium chrysogenum* are ubiquitous in outdoor and indoor environments and are inhaled throughout most peoples' lives. A previous study by our laboratory determined that high levels of inhaled *P. chrysogenum* conidia (1×10^4) similar to levels people would be exposed to in contaminated buildings induced high IgE and IgG1 antibodies specific to conidia antigens correlating with airway IL-4 production in C57BL/6 mice. Mice exposed to high numbers of conidia also developed significant airway eosinophilia correlating with increased IL-5 production. Mice were also exposed to similar numbers of nonviable conidia which would not germinate in vitro, and these mice did not develop airway inflammation or increased levels of IgE or IgG1 [9]. Nonviable conidia were utilized as a negative control in these studies since wild-type fungal conidia in the environment retain some viability despite the effects of UV light or other environmental factors. The effects of prolonged inhalation of ambient levels of viable conidia or spores have not been thoroughly evaluated.

We wanted to determine the effects of low doses of viable *P. chrysogenum* conidia with low viability inoculated by intranasal instillation (IN) into C57BL/6 mice. It was important to establish if ambient levels of conidia found in normal indoor environments would induce similar effects in the same murine model as examined in the high conidia number study. Previous studies by our laboratory determined that normal indoor environments contain less than 10 viable *Penicillium* conidia/m³ of air, and given that humans would inhale approximately 200 viable conidia/h, a person would be exposed to approximately 3×10^3 viable conidia in a normal indoor environment during a 40-hour workweek [7]. Deposition and clearance studies determined that approximately 4% of viable conidia inoculated in C57BL/6 mice were deposited in the alveolar spaces [10]. Since the lung capacity of mice is about 500-fold less than humans, we chose to inoculate mice with 1×10^2 viable conidia which would deliver approximately 4 viable conidia weekly to the mice.

In addition, preliminary studies indicated the presence of proteins with proteolytic activity in lyophilized extracts from viable *P. chrysogenum* conidia. Therefore, further studies were required to characterize these proteolytic

extracts and determine their roles in the allergic effects induced by viable *P. chrysogenum* conidia. One purpose of this study was to characterize the effects of *P. chrysogenum* conidia and the proteolytic enzymes released by viable *P. chrysogenum* conidia in a murine model. We also wanted to determine if the protease extracts alone would induce allergic effects similar to those induced by viable conidia in a murine model, and if these effects would be altered by challenge with viable or nonviable conidia. We chose to utilize the C57BL/6 model because of previous studies indicating its usefulness in characterizing in vivo responses to protease allergens and fungal conidia compared with BALB/c mice [9, 11].

The results of this study determined that low levels of viable *P. chrysogenum* conidia do not induce detectable responses in mice. This study also showed that viable *P. chrysogenum* conidia release protease allergens that induce specific responses in mice, including increased IgE and IgG1 as well as airway inflammation characterized by eosinophils and neutrophils.

Materials and Methods

Animals

Female BALB/c and C57BL/6 mice were ordered at 3 weeks of age from Charles River and housed in the Laboratory Animal Resource Center (LARC) at Texas Tech University Health Sciences Center (TTUHSC) in strict accordance with Institutional Animal Care and Use Committee (IACUC)-approved protocols. Animals were housed in an isolated room in the LARC in suspended stainless steel cages contained within a continuous high efficiency particle arrestor-filtered environment. Sterilized water and rodent chow were available ad libitum.

Conidia

P. chrysogenum samples utilized in this study were isolated from a building characterized with SBS [7]. To retain wild-type characteristics, conidia from this organism were suspended in phosphate-buffered saline (PBS) pH 7.0 and added to sterile pieces of cellulose-based ceiling tiles and incubated at 22°C for 7 days. The conidia were collected and stored desiccated under vacuum. For inoculations, stock conidia were resuspended in PBS and propagated on potato dextrose agar plates, pH < 7.0, as described previously [9]. Conidia were rendered nonviable by overnight incubation with filter paper soaked with formalin. Nonviable conidia plated on potato dextrose agar and incubated as described above produced no viable colonies.

Conidia Protease Extract Preparation

Viable *P. chrysogenum* colonies were propagated as described above. Conidia were collected in PBS, counted, and diluted to a concentration of 1×10^7 /ml in 0.1 M acetate buffer pH 4.0 with 0.01% w/v glucose. The suspended conidia were stirred at room temperature for 48 h, filtered, and the remaining solution was frozen and lyophilized. The dried product was dialyzed against 0.1 M acetate

Table 1. Treatment protocol for C57BL/6 and BALB/c mice with low levels of conidia and OVA

Group	Description of treatment	Dose of treatments
1	PBS IN weekly for 11 weeks	50 µl PBS IN weekly
2	Agarose beads IN weekly for 11 weeks	1 × 10 ² agarose beads in PBS IN
3	OVA IP primary dose, OVA IN weekly doses for 10 weeks	100 µg OVA/alum IP 100 µg OVA IN weekly
4	OVA IN primary dose, OVA IN weekly doses for 10 weeks	100 µg OVA IN 100 µg OVA IN weekly
5	OVA/agarose beads IN weekly for 11 weeks	100 µg OVA/1 × 10 ² agarose beads IN weekly
6	Nonviable conidia IP primary/IN weekly for 10 weeks	1 × 10 ² NV conidia/alum IP 1 × 10 ² NV conidia IN weekly
7	Nonviable conidia IN weekly for 11 weeks	1 × 10 ² conidia IN weekly
8	Viable conidia IP primary/IN weekly for 10 weeks	1 × 10 ² viable conidia/alum IP 1 × 10 ² viable conidia IN weekly
9	Viable conidia IN weekly for 11 weeks	1 × 10 ² viable conidia IN weekly

Table 2. Treatment protocol for IP sensitization of mice to protease extract (Pen ch) followed by IN inoculation with protease extract, 1 × 10⁴ viable (VIA) or nonviable (NV) *P. chrysogenum* conidia

Group	Description of treatment	Dose of treatment
PBS IP	PBS control IP weekly for 8 weeks	200 µl PBS/alum IP
PROT IP/PBS IN	Protease extract IP weekly for 6 weeks, PBS IN weekly for 2 weeks	20 µg protease/alum IP 50 µl PBS IN
PROT IP/IN	Protease extract IP weekly for 6 weeks, IN weekly for 2 weeks	20 µg protease/alum IP 20 µg protease IN
PROT IP/VIA IN	Protease IP weekly for 6 weeks, VIA conidia IN weekly for 2 weeks	20 µg protease/alum IP 10 ⁴ VIA conidia IN
PROT IP/NV IN	Protease IP weekly for 6 weeks, NV conidia IN weekly for 2 weeks	20 µg protease/alum IP 10 ⁴ NV conidia IN

buffer at 4°C for 48 h and rehyophilized. The sample was resuspended in PBS pH 7.4 and assayed for protease activity. The purity of the protease extracts was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [12]. Concentrations of the protease extracts were determined by Lowry protein assays [13]. Proteolytic activity of the viable conidia protease extracts, designated Pen ch, was determined by a trypsinogen activation assay [14]. The average specific activity of the extracts was 2,584 units/mg, which was only detectable at pH 4.0. Similar protease extracts were not isolated from nonviable conidia. The serine protease inhibitor leupeptin inhibited the extract proteolytic activity by almost 60%, while a protease inhibitor cocktail containing various serine, cysteine, aspartic, and metalloprotease inhibitors (Sigma) completely inhibited the proteolytic activity of the extract (unpubl. data).

Inoculations

For IN inoculations, each animal was lightly anesthetized using isoflurane, received 25 µl of the appropriate solution into each nare, and was held upright for 2 min after inoculations. For intraperitoneal

(IP) injections, ovalbumin (OVA), conidia, or protease extract were mixed with alum (Imject, Pierce) 1:1, and 200 µl of the antigen/alum solution were injected into the peritoneal cavity of mice using a hypodermic syringe. Each animal was inoculated with the final weekly dose 24 h prior to sacrifice.

Low-Dose Conidia Inoculations. A diagram of the inoculation scheme for the inoculation of low levels of viable conidia is shown in table 1. Five animals were assigned to each experimental group, were matched according to weight, and were housed as described previously. Both C57BL/6 and BALB/c mice were utilized in these studies. The standard research allergen OVA (Sigma) was utilized as a positive control. Agarose beads (Polysciences, Washington, Pa., USA) with average diameters of 1–3 µm were utilized in this study as a negative control to mimic the size of *P. chrysogenum* conidia.

Protease Extract and Conidia Inoculations. Female C57BL/6 mice 4–6 weeks old were matched and separated by weight (n = 8). The inoculation scheme is shown in table 2. Control mice were injected IP with either 200 µl PBS/alum once each week for 8 weeks (PBS IP) or with 20 µg protease extract/alum for 6 weeks followed by

two weekly IN inoculations with PBS only (PROT IP/PBS IN). Remaining groups of mice were injected IP with 20 µg protease extract/alum for 6 weeks followed by two weekly IN inoculations of either 20 µg protease extract, 1×10^4 viable (average viability = 25%) or nonviable conidia.

Bronchoalveolar Lavage and Preparation of Cytospin Slides

Each animal was sacrificed less than 24 h after the final inoculation by overdose with anesthetic and cardiac puncture. Sera were collected from blood drawn from each animal and stored at -20°C until analysis. Bronchoalveolar lavage (BAL) was performed with 4 ml Hanks balanced salt solution (Sigma) at 0.5-ml intervals. The lungs were removed and placed into neutral buffered formalin without inflation for histopathological analysis. BAL fluid was centrifuged, filtered, and stored at -20°C for ELISA analysis. BAL cells were resuspended and pelleted onto microscope slides by a Cytospin centrifuge (Shandon). Cells were stained with Wright-Giemsa and cell differential counts were conducted to differentiate inflammatory cells present in BAL fluid.

ELISA Analysis of BAL Fluid and Serum

Levels of cytokines and chemokines in BAL fluid and total serum immunoglobulin isotypes were analyzed using monoclonal antibody-based sandwich ELISAs. ELISA sets or matched-pair antibodies for IgE, IgG1, IgG2a, TNF- α , IL-4, IL-5, IL-6, IL-10 and IFN- γ were purchased from BD Pharmingen (San Diego, Calif., USA), and matched-pair antibodies for IL-13, MIP-2, KC, and eotaxin were purchased from R&D Systems (Minneapolis, Minn., USA). Briefly, monoclonal antibodies specific for each factor were bound to an immunoplate (Maxisorp, Nunc, Denmark), followed by nonspecific blocking with 0.1 M PBS pH 7.2 with 1% bovine serum albumin (BSA). A biotinylated-detecting antibody specific for the factor being assayed was added followed by incubation with HRP-conjugated streptavidin diluted 1:2,500 (Zymed, South San Francisco, Calif., USA). After a final washing step, the substrate tetramethylbenzidine (TMB; DAKO, Carpinteria, Calif., USA) was added and the reaction was stopped by the addition of 1 N H_2SO_4 . Absorbance readings were determined by a spectrophotometric plate reader with a 450-nm filter. The levels of each factor were determined from a standard curve.

Protease Extract-Specific ELISA

Ten micrograms of the protease extract Pen ch, 1×10^6 viable *Pc* spores, and 10 µg BSA diluted in PBS pH 7.2 were added to immunoplates (Maxisorp) and incubated overnight at 4°C to allow antigens to bind. The plates were blocked with 1% BSA for 1 h, and then antisera from protease extract-sensitized animals were added for 2 h at room temperature. After washing with PBS containing 0.01% Tween 20, the plates were incubated with a detecting antibody consisting of anti-mouse IgG1 conjugated with biotin for 1 h. After washing, streptavidin-HRP was added for 30 min. After subsequent washing, TMB was added, and the reaction was stopped by addition of 1 N H_2SO_4 . The absorbance readings of each well were quantified using a plate spectrophotometer with a 450-nm filter, and antigen-bound absorbance values were corrected for background BSA absorbance.

Analysis of Lung Sections

Formalin-preserved lungs were embedded in paraffin, sectioned, and mounted on slides for microscopic analysis. The tissue was stained with hematoxylin and eosin (H&E) to differentiate the spe-

cific cells and inflammation present. Each slide was also stained with periodic acid-Schiff to detect the production of mucin. Each specimen was labeled in a manner that blinded the pathologist to prevent observational bias. The lungs were examined at low and high power to determine the presence of any lesions or inflammation. A semi-quantitative numerical score was given to describe the degree of inflammation in various parts of each specimen: 0 = normal, 1 = few cells noticeable at low power, 2 = diffuse rings of cells noticeable only at higher power, 3 = diffuse to numerous rings of cells visible at low power, 4 = numerous cells visible at low power throughout tissue.

Statistics

Statistical analyses were performed by using Sigma Stat, a statistical software program designed by Jandel (SPSS). Data were analyzed for one-way analysis of variance (ANOVA), and the post hoc Tukey test was applied to determine which groups had significant differences compared to controls. Statistical significance was determined with $\alpha = 0.05$.

Results

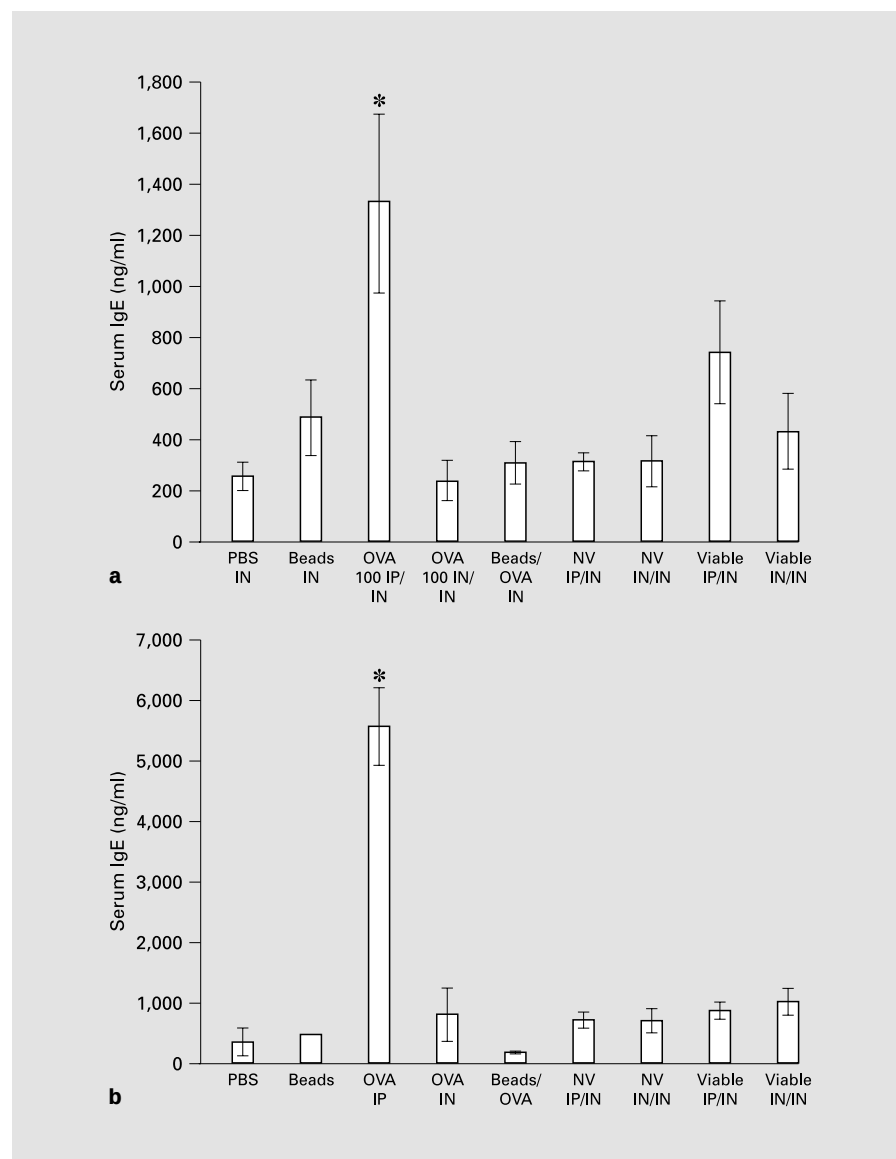
Exposure of C57BL/6 and BALB/c Mice to Low Levels of Conidia

Animals were inoculated IP and IN with OVA, 1×10^2 agarose beads, 1×10^2 viable or nonviable *P. chrysogenum* conidia for a period of 11 weeks. No significant increases in serum IgE (fig. 1), IgG1, or IgG2a (data not shown) from BALB/c or C57BL/6 mice to 1×10^2 viable *P. chrysogenum* conidia were detected during 11 weeks of treatment. C57BL/6 and BALB/c mice showed increased levels of IgE (fig. 1) and eosinophils (unpubl. data; $p < 0.05$) after 11 weeks of treatment with 100 µg OVA IP and IN. BAL fluid from treated animals was examined by ELISA for various cytokines. No significant production of lung cytokines was detected in any of the treatment groups (unpubl. data). No significant inflammation was seen in any of the spore treatment groups (unpubl. data).

IgE and Antigen-Specific IgG1 Responses of Protease-Sensitized Mice

C57BL/6 mice were sensitized for 6 weeks by IP injections of 20 µg of protease extract Pen ch adsorbed to alum as described in Materials and Methods and table 2. Challenge with protease extract (PROT IP/IN) and viable conidia (PROT IP/VIA IN) for 2 weeks produced significant increases in serum IgE (fig. 2a) and IgG1 (fig. 2b) ($p < 0.005$) as compared with protease-sensitized mice challenged IN with PBS (PROT IP/PBS IN). Protease extract-specific IgG1 was also increased in mice sensitized to and challenged with Pen ch compared with IgG1 from animals treated with PBS only (fig. 2c, $p < 0.001$).

Fig. 1. Serum total IgE levels in C57BL/6 (a) and BALB/c (b) mice after 11 weeks of IN and IP inoculations with OVA, agarose beads, and 1×10^2 viable and nonviable *P. chrysogenum* conidia. Bars represent mean total serum IgE concentration ($n = 5$), and error bars represent SEM. * $p < 0.05$ compared with negative controls (PBS and beads).



Airway Eosinophils and Cytokines

Airway BAL cell counts (fig. 3) revealed significant eosinophilia ($p < 0.001$) in protease-sensitized mice intranasally challenged with protease (PROT IP/IN) and viable conidia (PROT IP/VIA IN). No groups of mice showed significant increases in airway neutrophils (fig. 3). No significant levels of lung cytokines or chemokines were detected (data not shown).

Histology of Protease Extract-Sensitized Mice

Protease extract-sensitized and challenged mice developed inflammation characterized by both eosinophils and neutrophils in the alveoli and small airways noted in fig-

ure 4b by the arrow. These mice also developed perivascular inflammation characterized by both eosinophils and neutrophils with scores from 2 to 4 (fig. 5, $p < 0.001$). Lung tissue from mice challenged with saline only showed no evidence of inflammation (fig. 4a, 5). In addition, protease extract-sensitized and challenged mice also developed increased mucin production as indicated by the magenta staining of most of the cells in the epithelium as shown in figure 4d compared with saline-treated control mice (fig. 4c). All other groups of protease-sensitized mice showed little or no evidence of inflammation either in the perivascular or peribronchial spaces (fig. 5).

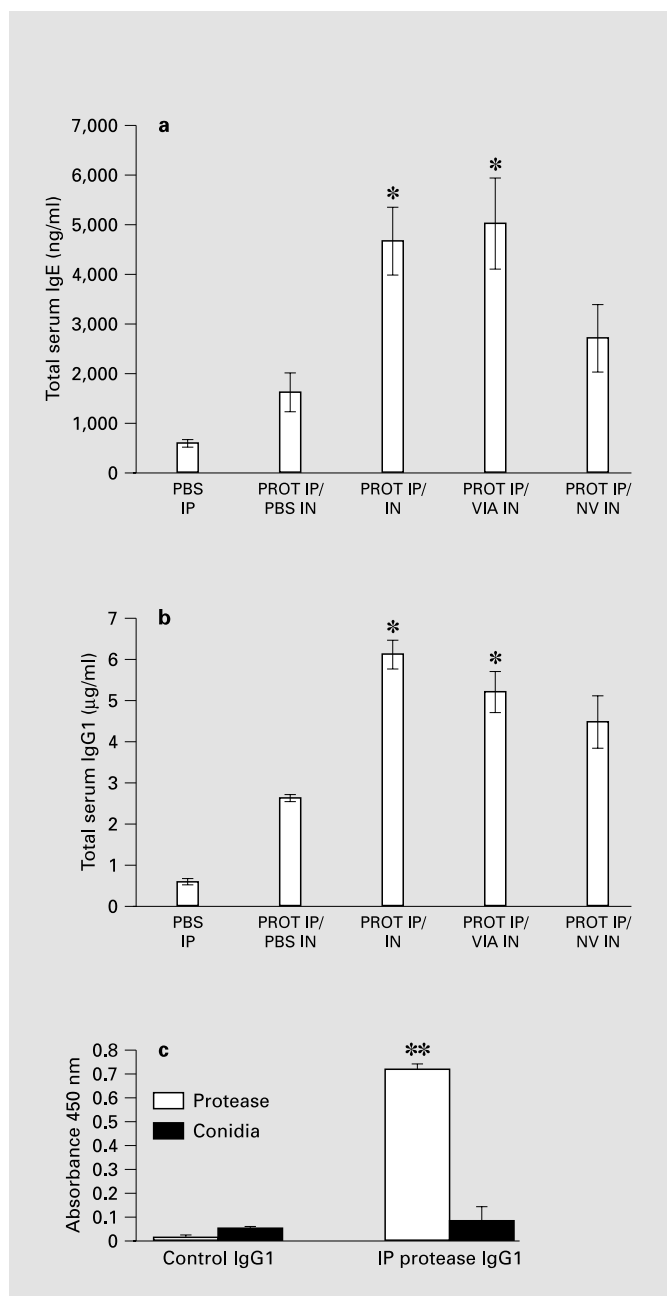


Fig. 2. Serum IgE and IgG1 levels and protease-specific IgG1 after 2 weeks of IN inoculations of protease-sensitized mice with Pen ch, 1×10^4 viable or nonviable *P. chrysogenum* conidia. Bars represent mean total serum IgE (a) and IgG1 (b) concentrations. * $p < 0.005$ compared with controls that received Pen ch IP weekly followed by IN challenge with PBS (PROT IP/PBS IN). c Absorbance. ** $p < 0.001$ protease extract-specific IgG1 (IP protease IgG1) compared with control sera (control IgG1). Error bars represent SEM.

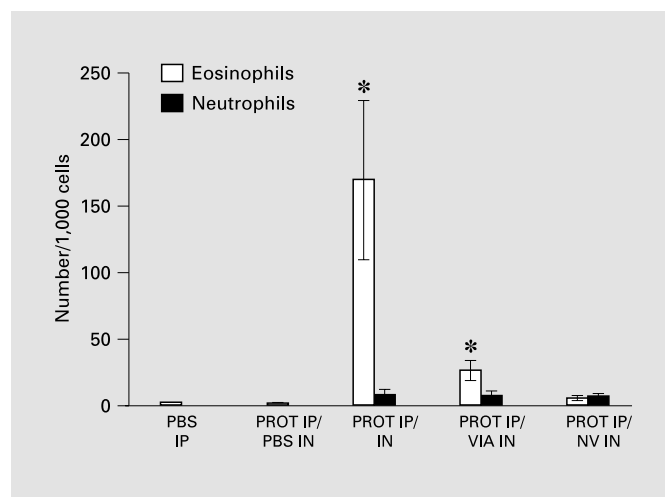


Fig. 3. Airway (BAL) eosinophils and neutrophils after 2 weeks of IN inoculations of protease-sensitized mice with Pen ch, 1×10^4 viable or nonviable *P. chrysogenum* conidia. Bars represent mean number of cells per 1,000 BAL cells counted ($n = 8$). * $p < 0.001$ compared with control mice (PROT IP/PBS IN). Error bars represent SEM.

Discussion

Numerous fungal conidia or spores are found in the outdoor air at any time and are impossible to prevent from entering buildings. Conidia less than $5 \mu\text{m}$ in size, which include *Penicillium* sp. conidia, are capable of being suspended in air for hours and can be inhaled into the lower respiratory tract [15]. There has been concern that long-term exposure to fungal conidia could lead to allergic or other aberrant immune responses. For the present study, we used *P. chrysogenum* conidia with relatively low viability and in numbers representing background levels found in indoor environments to inoculate different strains of mice to determine if they would become sensitized. An important finding in this study was that chronic exposure to ambient levels of viable *P. chrysogenum* conidia did not induce allergic effects in both C57BL/6 and BALB/c mice. Serum IgG2a and lung cytokines were not detected in significant amounts, and it was unclear what mechanism was responsible for the lack of allergic sensitization. The possibility exists that daily inoculations of 1×10^2 viable conidia could lead to sensitization. However, this type of exposure would be near impossible to perform due to development of tolerance to the inhaled anesthetics as well as difficulty in aerosolizing and delivering desired numbers of viable conidia by nose-only exposure. The chosen inoculation protocol was simi-

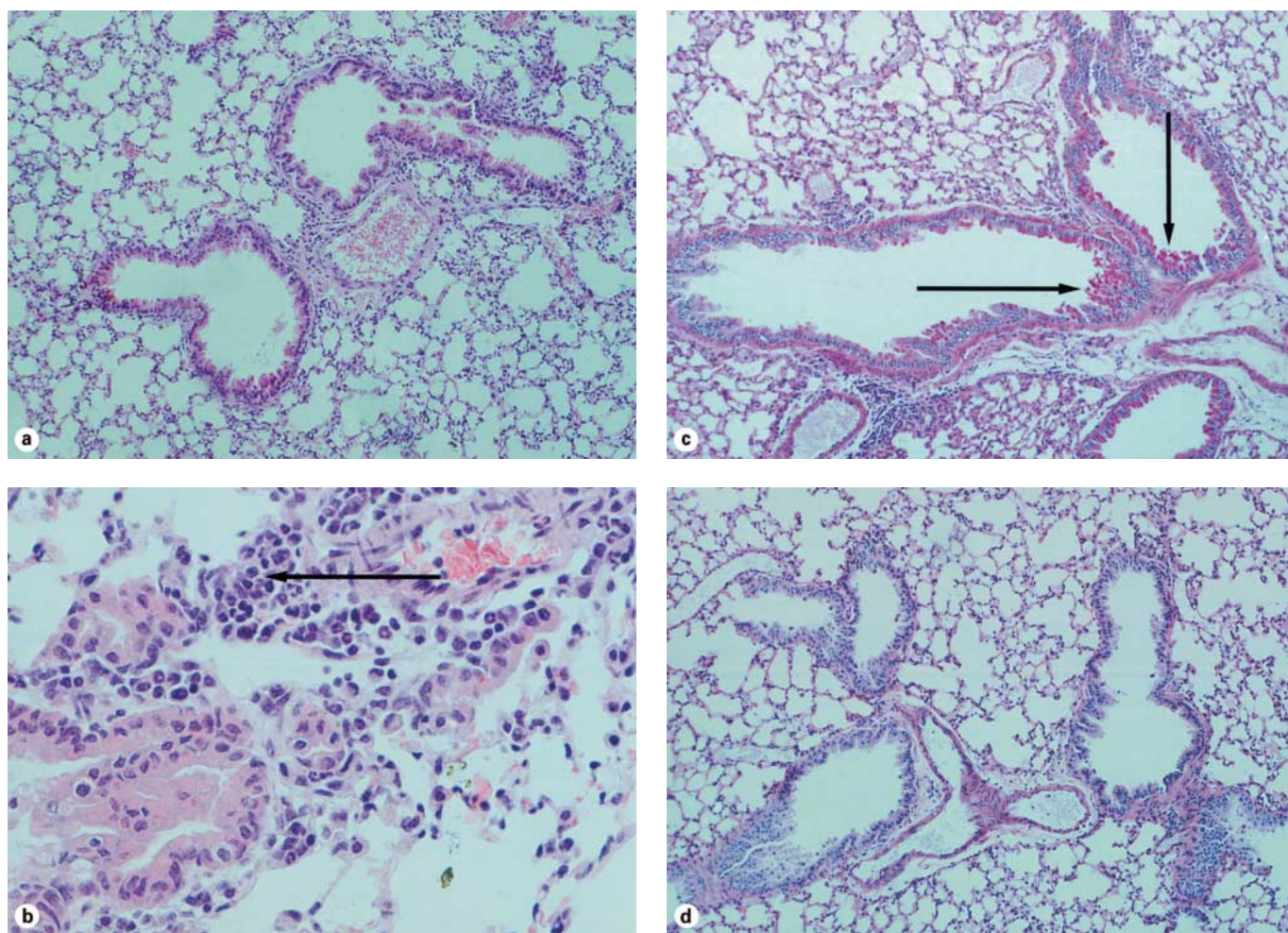


Fig. 4. Histopathological examination of lungs from mice sensitized for 6 weeks and challenged for 2 weeks with Pen ch. Significant perivascular and peribronchiolar eosinophilia and neutrophilia present in mice sensitized to and challenged with Pen ch (b) (H&E $\times$ 500) compared with mice treated with PBS only (a) (H&E $\times$ 125). Increased mucus cell hyperplasia in mice sensitized to Pen ch (c) (PAS $\times$ 125) compared with unsensitized control mice (d) (PAS $\times$ 125).

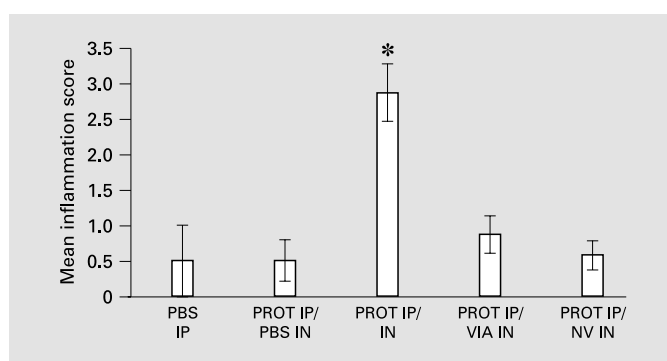


Fig. 5. Mean airway inflammation of mice sensitized to protease extract Pen ch. * $p < 0.001$ compared with PBS control (PROT IP/PBS IN). Error bars represent SEM. VIA = Viable; NV = nonviable.

lar to that used in the previous study where 1×10^4 viable conidia induced allergic sensitization in mice [9].

Conidia from several species of fungi, including *Penicillium* sp., are known to be associated with asthmatic patients [16]. Up to 68% of atopic adults and 20% of atopic children exhibit IgE specificity to *Penicillium* sp. extracts [17], and infants at high risk for developing asthma exposed to *Penicillium* sp. in their first year exhibited an increased risk for wheeze and persistent cough [18]. Several allergens have been characterized from various *Penicillium* sp. including *P. chrysogenum* as well as related *Aspergillus* sp. [17, 19, 20]. Few of these allergens have been characterized in animals models. Most in vivo studies have utilized conidia or allergenic extracts from the

common opportunistic pathogen *Aspergillus fumigatus* [21–23]. Although *A. fumigatus* is found in some buildings with occupant complaints, it is not as prevalent as *P. chrysogenum* [7, 8]. Therefore, we decided to focus our studies on characterizing the in vivo response to allergens isolated from viable conidia of *P. chrysogenum*.

We have presented evidence in this study of an extract, Pen ch, isolated from viable *P. chrysogenum* conidia with serine protease activity that induced allergic sensitization in C57BL/6 mice. The Pen ch protease extract allergens could be similar to previously characterized proteases from *P. chrysogenum* that have been shown to bind to IgE from human patients [17, 24, 25], but protease allergens from those studies were isolated from mycelial cultures and not from conidia alone. The conidia-specific protease extract Pen ch was also recognized by IgG1 from mice inoculated with 1×10^4 viable *P. chrysogenum* conidia in the previous study (unpubl. data). This would indicate the in vivo production or release of protease allergens by viable *P. chrysogenum* conidia. However, since the proteolytic activity of the extract is optimal at acidic pH, the proteolytic activity of the allergens is probably not important in the lung pathology in contrast to other characterized protease allergens [20, 26].

Mice sensitized to Pen ch and challenged with intranasal inoculations of Pen ch and 1×10^4 viable conidia produced significant levels of serum IgE and antigen-specific IgG1 as well as significant airway eosinophilia and neutrophilia. Since these pathological findings are consistent with a Th2-allergic response, the absence of systemic factors like Th2-specific cytokines and chemokines was

probably due to the detection limit of the ELISAs used. Mice sensitized to Pen ch and challenged with 1×10^4 nonviable *P. chrysogenum* conidia did not develop significant airway eosinophilia and neutrophilia or produce significant levels of serum IgE and IgG1. This confirms the absence of allergic effects by mice to nonviable conidia treated with methanol as reported previously by our laboratory [9]. A recent study showed that dendritic cells carry fungal antigens to lymph nodes for presentation to lymphocytes [27]. Without this presentation of antigen, airway eosinophil and neutrophil accumulation and antigen-specific serum IgE and IgG1 production associated with Th2 cytokines would not be expected to occur. The evidence presented in this study supports our hypothesis that chronic exposure to ambient levels of viable *P. chrysogenum* conidia do not induce allergic sensitization in mice, and that viable *P. chrysogenum* conidia release protease allergens that are important in sick building-related allergic symptoms associated with *P. chrysogenum* contamination. It is possible that only long-term exposure to these allergens due to contamination of buildings with high numbers of conidia would induce allergic symptoms in susceptible individuals. Future studies should be able to define the exact mechanism of allergic sensitization.

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