

An animal model for allergic penicilliosis induced by the intranasal instillation of viable *Penicillium chrysogenum* conidia

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Abstract

Background—A study was undertaken to determine the consequences of long term intranasal instillation of *Penicillium chrysogenum* propagules in a mouse model.

Methods—C57 Black/6 mice were inoculated intranasally each week for six weeks with 10^4 viable and non-viable *P chrysogenum* conidia. Cytokine levels and cellular responses in these animals were then measured.

Results—Compared with controls, mice inoculated intranasally each week for six weeks with 10^4 *P chrysogenum* conidia (average viability 25%) produced significantly more total serum IgE (mean difference 1823.11, lower and upper 95% confidence intervals (CI) 539.09 to 3107.13), peripheral eosinophils (mean difference 5.11, 95% CI 2.24 to 7.99), and airway eosinophilia (rank difference 11.33, 95% CI 9.0 to 20.0). With the exception of airway neutrophilia (mean difference 20.89, 95% CI 3.72 to 38.06), mice inoculated intranasally with 10^4 non-viable conidia did not show significant changes in total serum IgE, peripheral or airway eosinophils. However, when compared with controls, this group (10^4 non-viable) had a significant increase in total serum IgG_{2a} (mean difference 1990.56, 95% CI 790.48 to 3190.63) and bronchoalveolar lavage (BAL) fluid levels of interferon (IFN)- γ (mean difference 274.72, 95% CI 245.26 to 304.19). In addition, lung lavages from mice inoculated intranasally with 10^4 viable *P chrysogenum* conidia had significantly increased levels of interleukin (IL)-4 (mean difference 285.28, 95% CI 108.73 to 461.82) and IL-5 (mean difference 16.61, 95% CI 11.23 to 21.99). The IgG_{2a}/IgE ratio and the IFN- γ /IL-4 ratio was lower in the group of mice inoculated intranasally with 10^4 viable conidia than in the 10^4 non-viable conidia group and the controls. When proteins were extracted from *P chrysogenum* conidia, attached to microtitre plates and incubated with serum from the 10^4 viable group, significant increases in conidia-specific IgE and IgG_{2a} were observed compared with controls, while serum from the 10^4 non-viable group was similar to controls.

Conclusions—These data suggest that long term inhalation of viable *P chrysogenum* propagules induces type 2 T helper

cell mediated (Th2) inflammatory responses such as increases in total and conidia-specific serum IgE and IgG_{2a}, together with BAL fluid levels of IL-4 and IL-5 and peripheral and airway eosinophilia, which are mediators of allergic reactions.

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Keywords: *Penicillium chrysogenum*; conidia; cytokines; fungal allergy; sick building syndrome

Sick building syndrome, a layman's term for symptoms resulting from poor indoor air quality, has proved difficult to define and no single cause of the disease has been identified.¹ Complaints include rhinitis, difficulty in breathing, headaches, flu-like symptoms, and watering of the eyes, which suggests both upper and lower respiratory tract diseases.^{2,3}

In a previous study we have shown that *Penicillium* species (especially *P chrysogenum*) and *Stachybotrys* species are strongly associated with sick building syndrome.⁴ These studies also supported earlier findings that *Penicillium* species have become an important indoor contaminant.⁵ It has been shown that bronchial challenges with conidia of *Penicillium* species induced immediate and delayed type asthma in sensitised subjects.⁶ While these allergic reactions appear to result from the inhalation of fungal products, the mechanism(s) responsible for these phenomena remains unclear.

Allergic asthmatic reactions follow from the activation of aberrant allergen specific Th2 cells that induce excessive eosinophil and IgE production.⁷ During the last decade, research has focused on the development of T cells to interferon (IFN)- γ producing Th1 cells which promote murine immunoglobulin class switching to IgG_{2a}, or to the interleukin (IL)-4 producing Th2 cells which promote immunoglobulin class switching to IgE.⁸ In allergic diseases T cell activation and IL-4 production follow the presentation of allergens by antigen presenting cells (APC) to T cells.⁹ Th2 differentiation enhances production of Th2 cytokines such as IL-4 and IL-10 and down-regulates macrophage function and Th1 activation.¹⁰ Further T/B cell cooperation leads to the production of IgE. Interaction of specific IgE antibodies on the surface of effector cells (mast cells and basophils) with allergen triggers the early phase of immediate type hypersensitivity responses. These events initiate the development of allergic inflammation and are further characterised by infiltration of

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eosinophils and release of eosinophil components (eosinophil cationic protein, eosinophil peroxidase and the eosinophil major protein), followed by all the signs and symptoms of an inflammatory process. An important linkage between sensitisation and inflammation is represented by T cells that secrete mediators not only involved in IgE synthesis (IL-4), but also responsible for eosinophil recruitment (IL-5).¹¹⁻¹⁵

Little is known about the role of fungal propagules in the pathogenesis of allergic diseases and as trigger factors for clinical signs of allergic disease. This study was designed to examine the consequences of the introduction of *P chrysogenum* conidia into the mammalian lung. Because of the importance of *P chrysogenum* conidia in sick building syndrome,⁴ we have developed an animal model to determine the consequences of nasal instillation of these conidia. In an earlier study we measured the deposition, clearance, and retention of *P chrysogenum* conidia that were instilled intranasally in a mouse model.¹⁶ In that report we showed that the acute instillation of viable *P chrysogenum* conidia induced inflammatory reactions in a dose dependent manner, while the instillation of non-viable conidia did not. In this study we have examined the consequences of long term instillation of *P chrysogenum* conidia.

Methods

CONIDIA

The *P chrysogenum* culture employed in this study was isolated from a building that was experiencing sick building syndrome.⁴ *P chrysogenum* was grown on Sabouraud's dextrose agar (SDA), pH 5.6, at 22°C and 95% relative humidity for five days. The conidia were harvested by gentle washing with sterile endotoxin-free phosphate buffered saline (PBS). Some groups of conidia were harvested using absolute methanol. After five minutes in the methanol, all conidia were non-viable. All of the conidia were then washed twice by sedimentation (centrifugation at 10 000g for 15 minutes), resuspended with PBS and vortexed extensively. The conidia were sonicated for 10 s to disrupt clumping, filtered through sterile 20 µm cell filters (Falcon) to remove any remaining clumps or debris, and counted on a haemocytometer. This procedure yielded an average of 25% (±5%) viable singly dispersed conidia, averaging 3.5 µm in diameter (range 1–5 µm). The singly dispersed conidia (viable and non-viable) were adjusted in PBS to yield 2×10^5 conidia per ml. Serial dilutions were plated on SDA plates to determine the percentage viability of the conidia.

The allergenic components were extracted by hydrating the *P chrysogenum* conidia (approximately 1×10^{10}) in PBS containing 85 mM NaCl, 2.6 mM KH₂PO₄ and 49 mM Na₂HPO₄, pH 8.0, for one hour at 4°C with constant shaking. After centrifugation (10 000g for 15 minutes at 4°C), the conidia were sonicated for one hour at 4°C. The sonicated conidia solution was incubated for 36 hours at 4°C with constant shaking. After centrifugation (10 000g for 15 minutes at 4°C) to

pellet the conidia, the supernatant was dialysed against 20 volumes of 50 mM ammonium bicarbonate (two changes) for 48 hours at 4°C. After dialysis the supernatant was filtered (0.2 µm cellulose acetate) and the protein content of each extract was determined by the procedure of Lowry *et al.*¹⁷ The preparations were stored at -80°C.

ANIMALS

Female C57Bl/6 mice (Harlan), 5–6 weeks old, were maintained in a high efficiency particulate arrestor (HEPA) filtered room and in HEPA filtered suspended stainless steel cages, without bedding. Once a week for six weeks the mice were lightly anaesthetised (isoflurane and oxygen mixture), placed in an upright position, and inoculated intranasally with 50 µl (25 µl per nare) of PBS containing 1×10^4 non-viable or viable conidia (viability 25% (± 5%)). The mice were held in the upright position for two minutes to allow for complete instillation of the dose. Controls were inoculated intranasally with 50 µl (25 µl per nare) of PBS. Twenty four hours after the six week inoculation the mice were anaesthetised (halothane and oxygen mixture) and killed by cardiac puncture.

All animals were under the care of trained technicians and a full time veterinarian in the Laboratory Animal Resource Center. Animals were fed autoclaved feed and water ad libitum. All animals were treated in accordance with the policies established by the TTUHSC institutional animal care and use committee.

BLOOD AND LUNG LAVAGE

Samples for haematological examination were drawn by retro-orbital bleeding.¹⁸ The venous blood smear slides were stained with Wright-Giemsa stain and a cell differentiation and count (CDC) was performed. Total blood was also removed by cardiac puncture, processed to obtain serum, and stored at -80°C.

For the lung lavage, the trachea was exposed and a 22 gauge angiocatheter was inserted and tied in place with a suture. The lungs were lavaged (four times) by slowly instilling 1 ml of sterile endotoxin-free Hank's balanced salt solution (HBSS) at 37°C followed by gentle aspiration. The lungs were aseptically removed and placed in 10 ml of 10% neutral formalin without inflation. The total number of macrophages (viable and non-viable) in the bronchoalveolar lavage (BAL) fluid was determined using the trypan blue exclusion assay counted on a haemocytometer. The BAL fluid was centrifuged (1000g for 10 minutes at 4°C) to sediment the cells. The BAL cells were suspended in PBS to yield 2×10^5 macrophages per ml. Samples (200 µl) of the BAL cells were pelleted onto microscope slides using a cytospin centrifuge (Shannon). The cytospin slides were fixed in absolute methanol, stained with Wright-Giemsa stain, and a CDC was performed. The BAL supernatants were passed through 0.22 µm cellulose acetate filters and stored at -80°C.

CYTOKINE AND IMMUNOGLOBULIN ANALYSIS

The BAL supernatants were assayed for IL-4, IL-5, and IFN- γ . The amount of cytokines released was determined using sandwich ELISA specific for murine cytokines.¹⁵ Serum was assayed for total immunoglobulins (IgE, IgG₁, and IgG_{2a}) and conidia-specific immunoglobulins (IgG₁, IgE, and IgG_{2a}) using a sandwich ELISA specific for the appropriate murine antibody.¹⁹

Briefly, for the total serum or cytokine assays, flat bottomed 96-well Maxisorp microtitre plates (Nunc, Denmark) were coated overnight at 4°C with the appropriate capture antibody. Each plate was washed with PBS + 0.1% Tween 20 (PBS-T), pH 7.4, and blocked with Super Block /PBS (Pierce, Rockford, Illinois, USA). After four washes with PBS-T, eight twofold dilutions in duplicate of the appropriate control or standard and the test BAL fluid or serum were added to the respective wells. The plates were then incubated for two hours at room temperature, washed four times with PBS-T, and the appropriate biotinylated detecting antibody was added and incubated for one hour at room temperature. The plates were washed four times with PBS-T, avidin-horseradish peroxidase (HRP) was added, and the plates were incubated for one hour at room temperature. Plates were washed an additional four times with PBS-T and incubated with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) plus 30% hydrogen peroxide. After 20–30 minutes the HRP enzyme reaction was stopped by adding 2 mM sodium azide. Absorbance was read on a microtitre plate spectrophotometer (DynaTech MR 4000) at 410 nm. The optical densities of the unknown samples were interpolated using a standard curve (log 10–log 10).

Soluble proteins from *P chrysogenum* conidia were prepared as previously described above. The conidia-specific antibody assay was performed as above with the exception that the 96-well Maxisorp immunoplates were coated with 10 μ g/ml conidia-protein extract diluted in 0.1 M NaHCO₃ buffer, pH 8.2. After overnight incubation at 4°C the immunoplates were washed three times with PBS-T, pH 7.4, and blocked with Super Block/PBS for one hour at room temperature. The serum for each group was pooled and added undiluted to plates and incubated for two hours at room temperature. Absorbance was read on a microtitre plate spectrophotometer (DynaTech MR 4000) at 410 nm. The optical densities of the unknown samples were plotted as log 10 using a Sigma Plot computer program.

ALVEOLAR MACROPHAGES AND ELECTRON MICROSCOPY

To evaluate conidia-phagocyte interactions, pulmonary alveolar macrophages were obtained from the lungs of killed C57Bl/6 mice that had received intranasal inoculations of *P chrysogenum* conidia three, six, and 24 hours earlier. Lungs were lavaged with HBSS as described above. Cells were recovered by centrifugation at 1000g for 10 minutes. For transmission electromicroscopy, 1 \times 10⁵ alveo-

lar macrophages from mice that had received intranasal inoculations of viable *P chrysogenum* conidia were fixed in glutaraldehyde (3%) in 0.1 M sodium cacodylate buffer (pH 7.3) for one hour at room temperature. The samples were then post-fixed in osmium tetroxide (1%) and prepared for ultrastructural analysis by standard methods.²⁰

For the histopathological examinations the lungs were aseptically removed and fixed in buffered 10% formalin. Five μ m sections were prepared, stained with haematoxylin eosin and examined blind. The sections were evaluated for the presence of lung eosinophilia, foreign material, fibrosis, alveolar macrophages, and inflammatory cells.

DATA ANALYSIS

Statistical analysis ($\alpha = 0.05$) was performed using Sigma Stat, a statistical software package designed by Jandel (SPSS), to analyse the data using one way analysis of variance (ANOVA) of each group (10⁴ viable, 10⁴ non-viable, and controls). If the ANOVA demonstrated significance, a post hoc Tukey test (a modified *t* test) was used to make multiple comparisons to determine which group of mice had significant results compared with the control group. Normality was examined using the Shapiro-Wilk test. Any data that did not meet the assumption of normality were tested using the non-parametric Kruskal-Wallis one way analysis of variance on ranks. If the ANOVA on the ranks demonstrated significance, Dunn's test was used to make multiple comparisons to determine which group of mice had significant results compared with the controls. All data from the ELISA, with the exception of the conidia-specific immunoglobulins, were transformed log 10–log 10 according to the manufacturer's recommendations.

Results

SERUM IMMUNOGLOBULIN LEVELS AND BAL FLUID CYTOKINES

The mice (n = 6) inoculated with 10⁴ viable *P chrysogenum* conidia had a mean (SD) total serum IgE level of 2626.7 (1778.4) ng/ml, total serum IgG₁ level of 10 740.3 (523.0) ng/ml, total serum IgG_{2a} level of 3103.0 (1392.8) ng/ml, BAL fluid IL-4 level of 627.5 (187.1) pg/ml, BAL fluid IL-5 level of 49.8 (2.5) pg/ml, and BAL fluid IFN- γ level of 181.7 (62.7) pg/ml. The mice (n = 6) inoculated with 10⁴ non-viable conidia had a mean (SD) total serum IgE level of 833.3 (339.3) ng/ml, total serum IgG₁ level of 10 710.5 (562.9) ng/ml, total serum IgG_{2a} level of 4045.0 (545.1) ng/ml, a BAL fluid IL-4 level of 161.7 (92.1) pg/ml, BAL fluid IL-5 level of 0.5 (0.2) pg/ml, and BAL fluid IFN- γ level of 405.8 (29.1) pg/ml. The control group (n = 9) had a mean (SD) total serum IgE level of 803.6 (300.9) ng/ml, total serum IgG₁ level of 10 697.0 (1067.3) ng/ml, total serum IgG_{2a} level of 2054.4 (1272.8) ng/ml, BAL fluid IL-4 level of 342.2 (131.1) pg/ml, BAL fluid IL-5 level of 33.5 (0.9) pg/ml, and BAL fluid IFN- γ level of 131.1 (23.7) pg/ml.

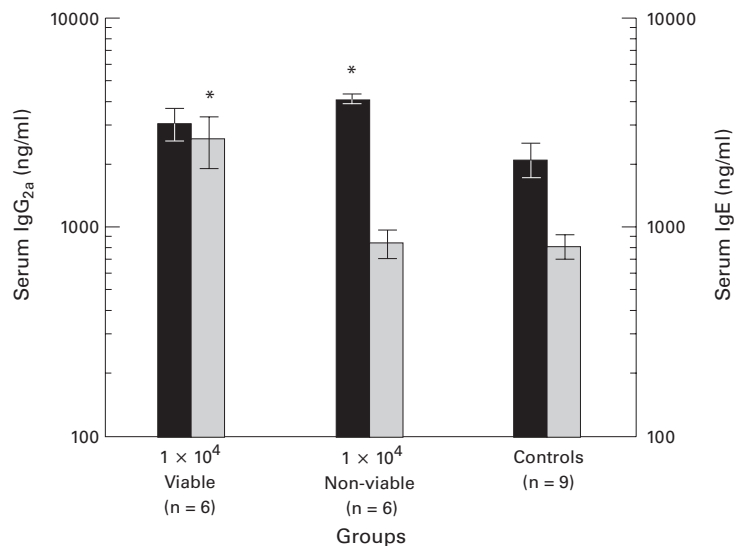


Figure 1 Serum levels of IgG_{2a} (solid bars) and total IgE (shaded bars) in mice inoculated intranasally with viable and non-viable *P chrysogenum* conidia once a week for six weeks. * $p < 0.05$ compared with controls. Error bars represent standard error of means (SE).

When compared with controls, mice inoculated with 10^4 viable *P chrysogenum* conidia had a significant increase in the total serum IgE ($p < 0.05$, mean difference 1823.11, lower and upper 95% confidence interval (CI) for difference of mean 539.09 to 3107.13) while the mice inoculated with 10^4 non-viable conidia group did not (fig 1). The intranasal instillation of 10^4 viable *P chrysogenum* conidia induced significant increases in BAL fluid levels of IL-4 ($p < 0.05$, mean difference 285.28, 95% CI 108.73 to 461.82) compared with the controls which strongly correlated with the increases in total serum IgE (fig 2).

In contrast, the total serum IgG_{2a} levels were significantly higher in the group inoculated with 10^4 non-viable *P chrysogenum* conidia ($p < 0.05$, mean difference 1990.56, 95% CI 790.48 to 3190.63) compared with controls,

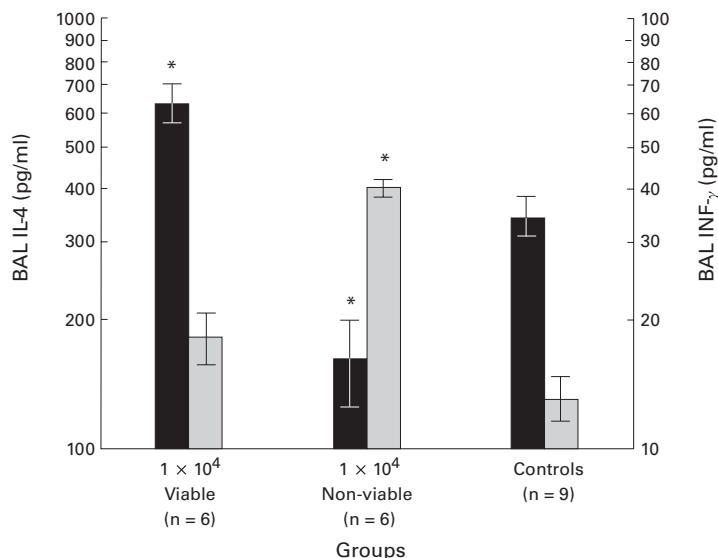


Figure 2 BAL fluid levels of IL-4 (solid bars) and IFN- γ (shaded bars) in mice inoculated intranasally with viable and non-viable *P chrysogenum* conidia once a week for six weeks. * $p < 0.05$ compared with controls. Error bars represent standard error of means (SE).

while the group inoculated with viable *P chrysogenum* conidia showed no significant increase in IgG_{2a} levels (fig 1). The increase in total serum IgG_{2a} in those inoculated with non-viable conidia also correlated with a significant increase in BAL fluid levels of IFN- γ ($p < 0.05$, mean difference 274.72, 95% CI 245.26 to 304.19 compared with controls; fig 2). There were no significant changes in the total serum IgG₁ levels.

The IgG_{2a}/IgE ratio in the group instilled intranasally with 10^4 viable *P chrysogenum* conidia was 1.18 compared with 4.86 and 2.56 in the group inoculated with 10^4 non-viable *P chrysogenum* conidia and the control group, respectively. In addition, the IFN- γ /IL-4 ratio in the group inoculated with 10^4 viable *P chrysogenum* conidia was 0.29, compared with 2.52 and 0.38, respectively, in the group instilled intranasally with 10^4 non-viable *P chrysogenum* conidia and the controls.

CONIDIA-SPECIFIC IMMUNOGLOBULINS

Mice inoculated with 10^4 viable *P chrysogenum* conidia had a mean (SD) conidia-specific serum IgE absorbance at 410 nm of 0.830 (0.002), a mean conidia-specific serum IgG₁ absorbance of 0.284 (0.0135), and a mean conidia-specific serum IgG_{2a} absorbance of 0.051 (0.001). Mice inoculated with non-viable conidia had a mean (SD) conidia-specific serum IgE absorbance at 410 nm of 0.039 (0.006), a mean conidia-specific serum IgG₁ absorbance of 0.123 (0.003), and a mean conidia-specific serum IgG_{2a} absorbance of 0.0505 (0.0035). The control group had a mean (SD) conidia-specific serum IgE absorbance at 410 nm of 0.0525 (0.0055), a mean conidia-specific serum IgG₁ absorbance of 0.140 (0.0045), and a mean conidia-specific serum IgG_{2a} absorbance of 0.069 (0.004).

Compared with controls, mice inoculated with 10^4 viable *P chrysogenum* conidia had a significant increase in the conidia-specific serum IgE ($p < 0.05$, mean difference 0.0305, 95% CI 0.021 to 0.040) while those inoculated with non-viable conidia had a significant decrease ($p < 0.05$, mean difference -0.0135, 95% CI -0.027 to -0.0005; fig 3). The intranasal instillation of 10^4 viable *P chrysogenum* conidia also induced significant increases in conidia-specific IgG₁ ($p < 0.05$, mean difference 0.145, 95% CI 0.122 to 0.168) compared with the controls, which correlated with the conidia-specific increases in serum IgE levels (fig 3).

In contrast, the conidia-specific serum IgG_{2a} levels were significantly lower in both the group inoculated with viable *P chrysogenum* conidia ($p < 0.05$, mean difference -0.0180, 95% CI -0.025 to -0.011) and those inoculated with non-viable *P chrysogenum* conidia ($p < 0.05$, mean difference -0.0185, 95% CI -0.027 to -0.010) compared with the controls (fig 3).

PERIPHERAL EOSINOPHILS

Intranasal instillation of 10^4 viable *P chrysogenum* conidia induced a significant peripheral blood eosinophilia ($p < 0.05$, mean difference 5.11, 95% CI 2.24 to 7.99) in these animals.

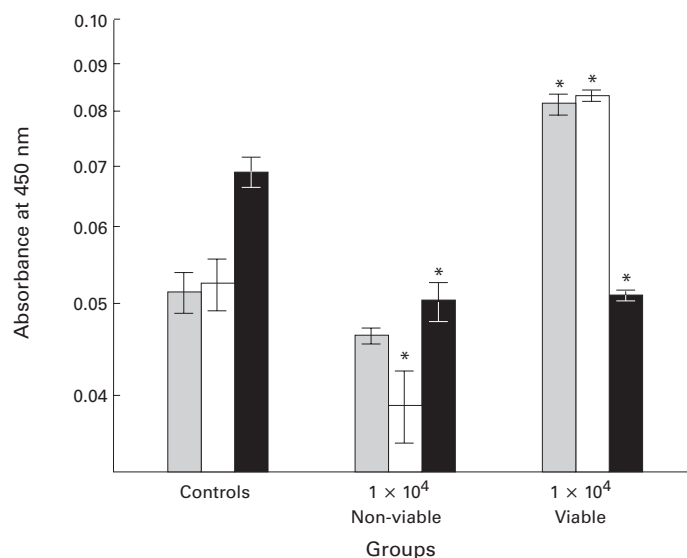


Figure 3 Levels of conidia-specific IgG₁ (shaded bars), IgE (white bars), and IgG_{2a} (solid bars) in pooled serum samples from mice inoculated intranasally with viable and non-viable *P chrysogenum* conidia once a week for six weeks. * $p < 0.05$ compared with controls. Error bars represent standard error of means (SE).

The group inoculated with viable *P chrysogenum* conidia had a mean (SD) peripheral eosinophil level of 10.3 (0.9) eosinophils per 100 white blood cells compared with 5.3 (0.8) eosinophils per 100 white blood cells in the control group and 7.3 (1.7) eosinophils per 100 white blood cells in the group inoculated with non-viable *P chrysogenum* conidia.

BAL FLUID CELLULAR RESPONSES

The group inoculated with 10⁴ viable *P chrysogenum* conidia had a mean (SD) BAL fluid eosinophil count of 14.17 (5.91) eosinophils per 1000 BAL cells compared with 0.22 (0.44) eosinophils per 1000 BAL cells in the control group and 0.5 (0.55) eosinophils per 1000 BAL cells in those inoculated with non-viable conidia. Figure 4 shows that intranasal instillation of 10⁴ viable *P chrysogenum* conidia induced a significant BAL eosinophilia ($p < 0.05$, rank difference 11.33, 95%

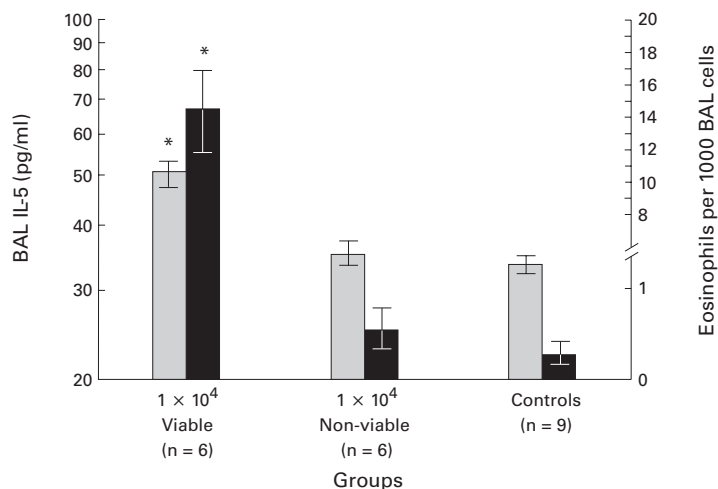


Figure 4 BAL fluid levels of eosinophils (solid bars) and IL-5 (shaded bars) in mice inoculated intranasally with viable and non-viable *P chrysogenum* conidia once a week for six weeks. * $p < 0.05$ compared with controls. Error bars represent standard error of means (SE).

CI 9.0 to 20.0) in the lungs of mice compared with the controls. The number of eosinophils in the group inoculated with non-viable conidia was not significantly different from that in the control group.

Figure 4 also shows that, compared with the control group, mice inoculated with 10⁴ viable *P chrysogenum* conidia had a significant increase in BAL fluid levels of IL-5 ($p < 0.05$, mean difference 16.61, 95% CI 11.23 to 21.99). However, in this group the increase in BAL fluid levels of neutrophils was only of borderline significance ($p = 0.079$). Although the intranasal instillation of 10⁴ non-viable conidia induced a significant neutrophilia ($p < 0.05$, mean difference 20.89, 95% CI 3.72 to 38.06) in these animals compared with the controls, no significant increase was seen in BAL fluid levels of eosinophils. While the control mice had a background level of neutrophils (mean (SD) 3.11 (4.37) neutrophils per 1000 BAL cells), very few eosinophils were found in the BAL fluid (mean (SD) 0.22 (0.44) eosinophils per 1000 BAL cells).

HISTOPATHOLOGY AND ELECTRON MICROSCOPY

Histological examination of the lung tissue revealed a few peribronchial lymphoid aggregates with plasma cells and some minor infiltration of eosinophils into the lung tissue of mice instilled with 10⁴ viable *P chrysogenum* conidia (data not shown), while no eosinophils were seen in the other groups (data not shown). However, the histopathological evaluation of the lung tissue samples showed no evidence of fibrosis or tissue remodelling.

Figure 5 is a series of electron micrographs of alveolar macrophages taken from the BAL fluid of mice three, six, and 24 hours after intranasal instillation of 10⁴ viable *P chrysogenum* conidia. These micrographs show that the *P chrysogenum* conidia have been phagocytosed and are within phagosomes and in various stages of destruction. Figure 5D is a micrograph of a control *P chrysogenum* conidia from a preparation that had not been injected into mice, but that had been prepared for morphological examination as described above.

Discussion

This study of the intranasal instillation of *P chrysogenum* conidia into C57B1/6 mice has produced interesting findings. The results show that inoculation of viable *P chrysogenum* conidia into the lungs of these mice results in inflammatory reactions, and that sensitisation to viable *P chrysogenum* conidia via mucosal exposure is efficient and does not require the potentially immunomodulatory intraperitoneal or subcutaneous priming (with adjuvant) used in the ovalbumin models to evoke airway inflammation. It should be noted that most asthma mouse models use ovalbumin injected intraperitoneally into BALB/c mice. However, preliminary experiments conducted in our laboratory using BALB/c instilled intranasally with viable *P chrysogenum* conidia only induced airway neutrophilia and no increases in total serum IgE levels (data not shown). This is consistent with experiments conducted by Stewart

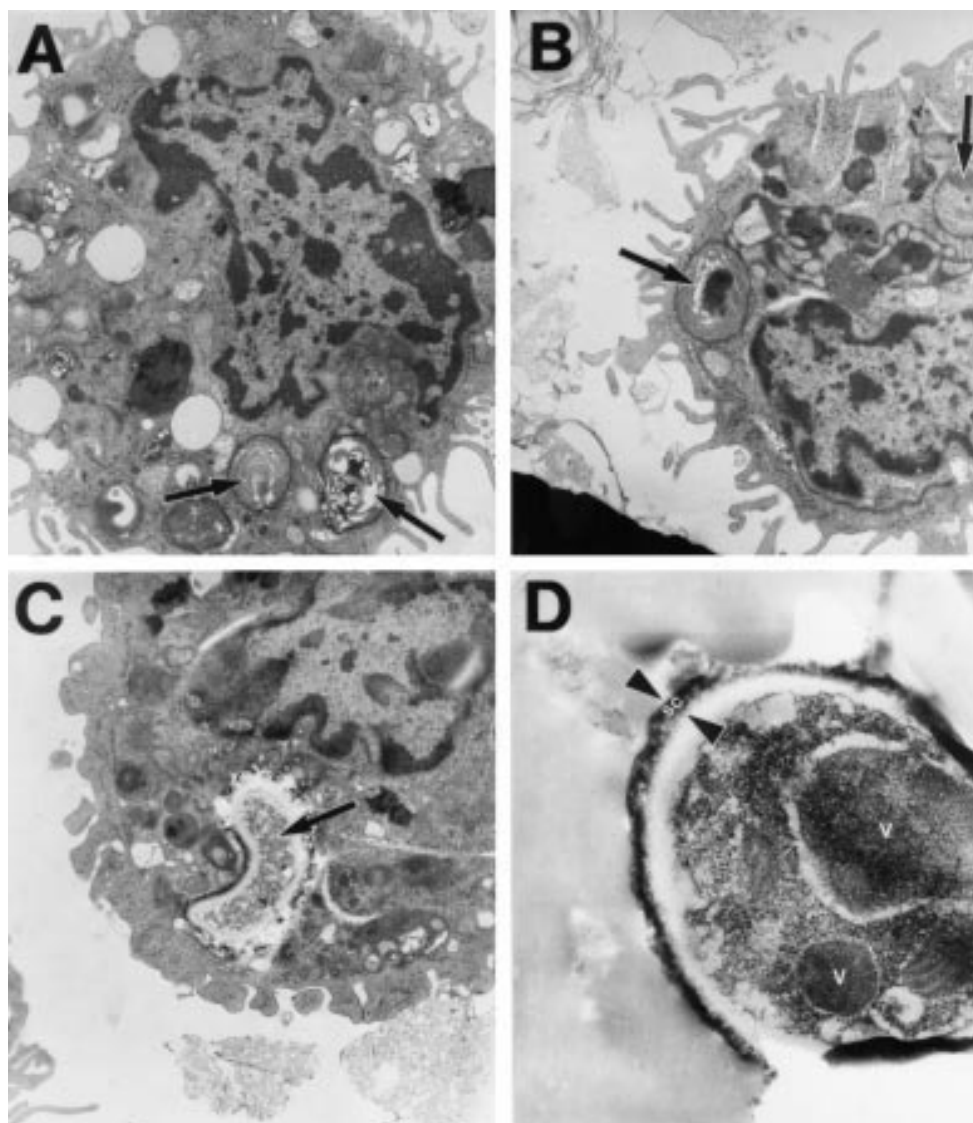


Figure 5 Ultrastructure of alveolar macrophages taken from the BAL fluid of mice (A) three hours, (B) six hours, and (C) 24 hours after instillation of viable conidia (magnification $\times 10\,000$). Phagocytosed conidia (arrows) at various stages of digestion were commonly observed within phagosomes of the macrophage at all the times studied. Temporal correlation of conidia destruction was not apparent as many macrophages contained conidia in various stages of breakdown, even after three hours. Residual bodies were present in cells at all times, typical of alveolar macrophages. (D) Ultrastructure of *P. chrysogenum* conidia (magnification $\times 43\,750$) before instillation (spore coat (sc) between the arrow heads and spore vacuoles (v)). This morphology is comparable to the minimally damaged instilled conidia captured in (C). The conidia in (A) and (B) are apparently in later stages of destruction.

et al who demonstrated that *Der p 1*, the major house dust mite antigen, induced an IgE response in C57Bl/6 mice but not in BALB/c mice.²¹ Long term exposure to 10^4 viable *P. chrysogenum* conidia caused a significant increase in total serum IgE, together with conidia-specific IgE and IgG₁. The significant increases in total IgE compared with controls indicate an allergic response.¹⁴ In addition, the same animals had significant peripheral and airway eosinophilia following long term exposure to 10^4 viable conidia. Significant increases in eosinophils, especially airway eosinophils, compared with controls indicate an allergic response and one of the most important indicators of asthma-like reactions in mammals.^{11 12 14 22} The cellular and IgE increases are supported by the significant increases in IL-4 and IL-5 in the BAL fluid of mice inoculated with viable conidia. These

cytokines are required for IgE synthesis and eosinophil recruitment, respectively. The production of these cytokines was measured 24 hours after intranasal inoculation. Although the BAL fluid levels of IL-5 in the mice inoculated with viable conidia were significantly different from the controls, we do not know if the production of this cytokine was increasing or decreasing at 24 hours. Nevertheless, the significant production of these cytokines (IL-4 and IL-5) in the lungs of mice inoculated with viable conidia, coupled with the airway eosinophilia, strongly suggests that the instilled viable conidia are inducing this reaction.

In addition, the IgG_{2a}/IgE and IFN- γ /IL-4 ratios, together with peripheral and airway eosinophilia, suggest that the inflammatory response observed in the group given viable *P. chrysogenum* conidia was Th2 mediated. This concept is supported by data showing that

proteins extracted from *P chrysogenum* conidia incubated with serum from the group inoculated with viable conidia were IgE-specific, while the serum from the group inoculated with non-viable conidia was not. In addition, even though the total serum IgG₁ levels were not significantly different in either of the groups compared with the controls, there was a significant increase in conidia-specific IgG₁ in the group inoculated with viable conidia while that in the group given non-viable conidia was similar to the control group.

In contrast, the IgG_{2a}/IgE and IFN- γ /IL-4 ratios, together with airway neutrophilia, imply that the inflammatory response observed in the group that received non-viable *P chrysogenum* conidia was Th1 mediated. However, the conidia-specific IgG_{2a} levels in both this group and the group inoculated with viable conidia was significantly less than in the control group.

These results do not discount the possibility that the viable conidia are producing an unknown protein that may be inducing these reactions. The extracted proteins used in the conidia-specific immunoglobulin ELISA were from both viable and non-viable conidia. It should also be noted that the viable conidia that were instilled into the animals consisted of 25% viable conidia and 75% non-viable conidia. Even though the data demonstrate a total serum and conidia-specific IgE and IgG₁ response for animals inoculated with viable conidia, the group that received non-viable conidia (100% non-viable) did not show any increases in conidia-specific IgE, IgG₁, or IgG_{2a}. However, the total serum IgG_{2a} and BAL fluid levels of IFN- γ from animals inoculated with non-viable conidia were significantly higher than those of the control group. We have previously reported that viable conidia could remain in the lungs long enough to produce substances that may induce allergic reactions.¹⁶ That earlier study showed that approximately 18% of the viable conidia instilled intranasally were deposited into the lungs and that, after nine hours, 4% of the viable conidia were recoverable from the lungs and were recoverable up to 36 hours after the initial inoculation.¹⁶ Those data also suggested that the remaining viable conidia were deposited and retained in the alveolar spaces where the primary clearance mechanism involves the pulmonary macrophages. The micrographs in the present study clearly show phagocytosis of *Penicillium* conidia and probable degradation. Our results suggest that viability of the conidia and the possible production of an unknown protein, and not some cell wall constituents (e.g. (1 \rightarrow 3)- β -glucans) may be responsible for the chronic Th2 mediated airway inflammation observed in fungal-induced allergies. This is because cell wall constituents such as (1 \rightarrow 3)- β -glucans are found in both non-viable and viable conidia. We are currently examining viable conidia for production of proteins during attempted germination.

Although a histological examination of the lung tissue revealed minor infiltration of eosinophils in the group given viable conidia, no lung tissue damage or remodelling was

observed. This might be due to the short duration of the study. Earlier studies (intranasal inoculations of viable *P chrysogenum* conidia once a week for three weeks) did not find any significant changes in airway levels of IL-5 or any airway eosinophils.¹⁶ This suggests that chronic airway inflammation in our model may require a longer exposure period to induce identifiable airway damage and remodelling.

This study presents additional information that the inhalation of fungal propagules (specifically *Penicillium* species) and their viability may play a role in eosinophilic bronchitis. This relationship could be causal or merely an exacerbation. Our earlier work showed that sick building syndrome and the dominance of *Penicillium* species are strongly associated.⁴ Other studies have suggested that there are associations between damp housing, chronic childhood respiratory symptoms, and sensitisation to house dust mites and mould allergens, including *Penicillium* species.^{23, 24} If further studies continue to show this same trend, it should be clear that *Penicillium* species conidia should be removed (to whatever degree possible) from the indoor air of houses and public buildings. This removal could theoretically result in a significant reduction in at least the exacerbation of fungal-induced sick building syndrome in developed countries.

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