Acute Inflammatory Responses to \textit{Stachybotrys chartarum} in the Lungs of Infant Rats: Time Course and Possible Mechanisms

Iwona Yike,*,†,1 Thomas G. Rand,‡ and Dorr G. Dearborn*‡

*Mary Ann Swetland Center for Environmental Health, and †Department of Pediatrics, Case Western Reserve University, Cleveland, Ohio 44106, and ‡Saint Mary’s University, Halifax, Nova Scotia, Canada B3H3C3

Received September 7, 2004; accepted December 1, 2005

\textit{Stachybotrys chartarum} has been linked to building-related respiratory problems including pulmonary hemorrhage in infants. The macrocyclic trichothecenes produced by \textit{S. chartarum} have been the primary focus of many investigations. However, in addition to trichothecenes this fungus is capable of producing other secondary metabolites and a number of protein factors. This study examines the effects of intact, autoclaved, and ethanol-extracted spores on the lungs of infant rats as an approach to differentiate between secondary metabolites and protein factors. Seven-day-old infant rats were exposed intratracheally to $1 \times 10^5$ spores/g body weight (toxic strain JS58-17) and sacrificed at various times up to 72 h. The inflammatory response was measured by morphometric analysis of the lungs and determination of inflammatory cells and cytokine concentrations in bronchoalveolar lavage (BAL) fluid. Alveolar space was greatly reduced in animals exposed to fungal spores compared to phosphate buffered saline (PBS)-treated controls. The largest effects were observed in pups treated with intact spores where alveolar space 24 h after treatment was 42.1% compared to 56.8% for autoclaved spores, 51.1% for ethanol-extracted spores, and 60.6% for PBS-treated controls. The effects of different spore preparations on inflammatory cells, cytokine, and protein concentrations in the BAL fluid can be ranked as intact > autoclaved > extracted. Tumor necrosis factor alfa (TNF-α), interleukin 1-beta (IL-1β), and neutrophils were the most sensitive indicators of inflammation. The difference between autoclaved (100% trichothecene toxicity, denatured/enzymatically inactive proteins) and intact (100% trichothecene activity, unaltered/released proteins) spores indicates the involvement of fungal proteins in the inflammatory response to \textit{S. chartarum} and sheds new light on the clinical importance of “nontoxic” strains.

Key Words: \textit{Stachybotrys chartarum}; trichothecenes; inflammatory response.

\textit{Stachybotrys chartarum} is a toxigenic mold found often in indoor air of buildings with moisture problems. Among the adverse health effects linked to the presence of this fungus is the “building-related illness,” which includes runny nose, cough, and irritation of the eyes and throat (Johanning et al., 1996). Exposure to \textit{S. chartarum} has been linked to an outbreak of pulmonary hemorrhage in infants (Dearborn et al., 2003; Etzel et al., 1998), although the etiology still remains unclear.

Macrocyclic trichothecenes produced by \textit{S. chartarum}, which are among the most potent protein synthesis inhibitors (Ueno, 1977), have become the primary focus of several investigations. However, in addition to trichothecenes, this fungus is capable of producing other secondary metabolites such as spirodrimanes and atranones (Jarvis, 2002) and a number of protein factors such as hemolysin (Vesper et al., 1999, 2001) and proteinases (Kordula et al., 2002; Yike et al., 2002), which may also contribute to disease onset.

Alcohol extraction of \textit{S. chartarum} spores leads to a sharp decrease in pulmonary inflammation in rats when compared to the effects of intact spores. We have previously reported that such extraction practically eliminates trichothecene toxicity of the spores (Yike et al., 2001). These results and the reports of Rao et al. (2000a,b) suggested that trichothecenes were the primary factors responsible for induction of inflammatory response in the lungs of animals exposed to \textit{S. chartarum}. However, alcohol extraction not only removes trichothecenes and other small molecules from the spores, but also denatures fungal proteins that may also contribute to pulmonary injury. Contrary to the earlier reports of Nikulin et al. (1996, 1997), we have observed strong inflammatory responses in infant rats when using strains of \textit{S. chartarum} that have low trichothecene content (Yike and Dearborn, 2004). Similar observations on mice were described by Flemming et al. (2004) and Leino et al. (2003). Other fungi that do not produce trichothecenes are also known to elicit inflammatory effects (Cooley et al., 1999), indicating the presence of other proinflammatory substances. The involvement of agents such as β-D-glucan in the inflammatory process is well documented (Rylander, 1999).

Proteolytic enzymes secreted by fungi have only recently been investigated as possible inducers of inflammation. It has been suggested that, in addition to their antigenic properties, fungal proteases act via protease-activated receptors and G proteins to activate production and release of proinflammatory cytokines (Kauffman et al., 2000). A serine protease from...
**S. chartarum** has been purified and characterized as having broad substrate specificity that would enable it to hydrolyze many of the physiologically significant proteins in the lung such as collagen, protease inhibitors, and neuropeptides (Kordula et al., 2002). We have shown variable proteolytic activity and the presence of serine proteases in the extracts from the spores of different isolates of *S. chartarum* (Yike et al., 2002).

Stachylysin, a hemolytic protein isolated from *S. chartarum*, may also contribute to tissue destruction and hemorrhage in the lung (Gregory et al., 2003; Vesper et al., 2001; Vesper and Vesper, 2002). The profile of toxin and hemolysin production by different strains of *S. chartarum* isolated from houses in Cleveland, OH, revealed that the strains originating from houses of patients suffering from pulmonary hemorrhage were likely to be highly hemolytic but did not always contain macrocytotoxic trichothecenes (Jarvis et al., 1998; Vesper et al., 1999).

In this study we examine the hypothesis that rapidly released trichothecene mycotoxins are primarily responsible for the acute changes in animal growth and proinflammatory cells and cytokines, while slowly released fungal proteins may be involved in the sustained lower level of inflammation. Comparing pathophysiological effects of spore preparations in which toxicity and ability to release biologically active proteins have been differentially altered allowed the assessment of the relative contribution of different classes of fungal components to the inflammation and lung injury in animals exposed to *S. chartarum*.

**METHODS**

**Experimental Design**

*Acute inflammatory effects of different spore preparations.* Four groups of 7-day-old Sprague-Dawley rat pups were used in these experiments. Fungal spores were delivered into the lungs via tracheostomy. One group was exposed to the intact, freshly harvested spores of *S. chartarum* strain JS 58–17 at 100,000 spores/g body weight (spg BW). Another group was treated with heat-inactivated (autoclaved) spores at the same dose. The third experimental group was treated with the same dose of ethanol-extracted spores, while the control group received phosphate buffered saline (PBS). The dose of 100,000 sp/g BW was selected based on our earlier studies (Yike et al., 2001), where it was found to cause significant lung inflammation without mortality and severe morbidity and allowed for reproducible measurements of inflammatory indices. The animals (twelve pups per time point) were sacrificed at 0, 3, 8, 16, 24, 36, 48, and 72 h following the instillation of spores. The lungs of six pups from each group were isolated, inflation fixed in 3% paraformaldehyde, and processed for histological evaluation. Bronchoalveolar lavages were performed on the remaining six pups and analyzed for cell content, protein, tumor necrosis factor alpha (TNF-α), and interleukin 1-beta (IL-1β) and urea.

*In vivo release of satratoxin G and stachylysin.* The release of satratoxin G into the lungs was studied in a separate group of animals exposed to 4 × 10⁵ spg BW (high trichothecene-producing isolate JS58-17, three animals per time point) and lavaged at 0, 15, 45, 60 min and 2, 3, 4, 5, 6, 16, and 24 h. A higher dose of fungal spores was chosen to facilitate toxin detection. Another group of rat pups exposed to 1 × 10⁶ spg BW of highly hemolytic isolate JS 58–06 (to facilitate stachylysin detection) and lavaged on day 1, 2, 3, 4, 7, 21, and 28 (three animals per time point) was used to study the release of stachylysin.

**Fungal spores.** Spores of *Stachybotrys chartarum* strain JS58-17, originally isolated from a home in Cleveland, Ohio, were collected from dry wall cultures as described before (Vesper et al., 1999), and their toxicity measured using the luciferase translation inhibition assay (Yike et al., 1999). Toxicity was expressed as Satratoxin G equivalents calculated by comparing the toxic effects of fungal spores with those of pure toxin. The viability of the spore preparations collected from dry wall cultures was evaluated by placing them on potato dextrose agar and counting colonies after 48-h incubation at 30°C. Fungal spores collected from dry wall cultures were suspended in PBS with 0.1% Tween 20, quickly counted, and immediately injected directly into the trachea of 7-day-old rats at a concentration of 1.0 × 10⁶ spg BW in a final volume of 20 μl. Extracted spores were prepared by suspending 2 × 10⁶ spores in 10 ml of 95% ethanol. The suspensions were kept for 16 h at room temperature, whereupon they were placed in an ultrasonic bath for 30 min, still at room temperature. Centrifuged spores were resuspended in 10 ml of fresh 95% ethanol and sonicated again. Spore pellets were then washed three times with 20 μl of PBS and resuspended in a small volume of PBS with 0.1% Tween 20, counted, and instilled as described below. Autoclaved spores were collected from dry wall cultures treated at 120°C for 15 min.

The release of stachylysin was studied using the spores of highly hemolytic isolate JS58-06 grown on dry wall.

**Animals.** Pregnant Sprague-Dawley female rats at 18–19 days of gestation were obtained from Charles River, (Wilmington, Massachusetts). The rats were housed in microisolators in the animal facility and fed the standard diet of Teklad 8664 (Harlan, Madison, Wisconsin) and water ad libitum. Each litter contained between 10–12 pups. The animal research protocol was received for compliance with the standards of humane treatment of animals and approved by the Case Western Reserve University’s institutional animal care committee.

**Instillation of fungal spores.** Seven-day-old newborn rats (mean weight 16.99 ± 0.23 g, SEM, n = 288) were anesthetized with methoxyflurane (Shering-Plough Animal Health Corp., Union, NJ). A transverse skin incision was made, and the trachea was exposed by blunt dissection, whereupon 1.0 × 10⁵ spg BW suspended in 20 μl of PBS were injected directly into the trachea using a 24 G catheter needle attached to a sterile Hamilton syringe. The incision was closed and treated with New Skin liquid bandage (Medtech Laboratories Inc., Jackson, WY) to facilitate healing and decrease maternal cannibalism. Control animals received 20 μl of PBS/Tween-20 solution.

**Histology.** Isolated lungs were inflation fixed at 20 cm H₂O in 3% paraformaldehyde-PBS solution for 48 h, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Lungs embedded in paraffin were sectioned onto a microscope slide and stained with Harris Haematoxylin and aqueous eosin (H&E). Control animals received 20 μl of PBS/Tween-20 solution.

**Morphometry.** Morphological changes in the lungs of infant rats were evaluated by morphometric analysis. This methodology has been used before to study the lungs of mice exposed to *S. chartarum* (Rand et al., 2003) and provided us with a quantitative measure of changes in alveolar air space due to granuloma formation in lungs inflamed as a result of exposure to fungal spores. Alveolar space was defined as the percentage of air space in the lung parenchyma. H&E-stained lung sections were used for quantitative analysis of alveolar space areas. Briefly, six animals were analyzed for each time point, and 10 regions of the lung parenchyma from each animal were randomly scanned using a 20x objective. Large airways and lung vessels were excluded. Randomly selected areas included granulomas with no alveolar space as well as sections of the lungs with very high percentage of air space.

An image field 4.18 × 10⁻⁴ m² was defined over a region of parenchyma entirely filling the field of view. With PC image analysis software from PCI Genomics, pixel intensity values were established that defined the alveolar space with elimination of all the cells and cellular debris within the spaces. The alveolar space area was then determined within each region of interest field (ROI) as percentage values.

In conclusion, the findings of this study confirm that the *S. chartarum* strain JS58-17 is a promising model to study the role of fungal spores in the development of pulmonary disease. The acute changes observed in our experiments suggest that the rapid release of trichothecene mycotoxins, particularly satratoxin G and stachylysin, plays a crucial role in the initial inflammatory response. Further studies are needed to elucidate the mechanisms by which these toxins contribute to lung injury and to identify potential targets for therapeutic intervention.
Characterization of Different Spore Preparations

<table>
<thead>
<tr>
<th>Spore Preparation</th>
<th>Toxicity^a</th>
<th>Proteolytic Activity</th>
<th>Hemolytic Activity</th>
<th>Viability %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>0.67 pg ± 0.12</td>
<td>0.41 ± 0.04</td>
<td>++++</td>
<td>100</td>
</tr>
<tr>
<td>Autoclaved</td>
<td>0.73 pg ± 0.15</td>
<td>ND^*</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Ethanol-extracted</td>
<td>≤1.0 × 10^{-5} pg</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
</tr>
</tbody>
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^a Determined by luciferase translation inhibition test; n = 5. ^* ND = not detected.

**Bronchoalveolar lavage (BAL).** The rats were weighed, and the total lung capacity (TLC, −60 μl/g body weight) (Sahebjami, 1992) was estimated for each animal. Anesthetized animals were exsanguinated via the right ventricle, and the lungs were perfused with PBS. The trachea was cannulated using a 24 G catheter attached to a 1-ml tuberculin syringe. The lungs were lavaged three times with a sterile PBS volume equal to 75% of TLC. Protease inhibitors, PMSF at final concentration of 0.1mM and ethylenediamine tetraacetic acid at final concentration of 5 mM, were added to the BAL fluid, and 200 μl aliquots of the BAL fluid were transferred into separate microfuge tubes for hemoglobin measurements. The remaining fluid was centrifuged for 10 min at 100 × g at 4°C. The supernatants were filtered through 0.22-μm syringe filters and stored at −80°C prior to cytokine and urea assays. The pellets were suspended in 1 ml of ice-cold PBS solution, and the cell count was determined using a hemocytometer. Aliquots of the cell suspension were transferred to slides in a cytospin centrifuge. Slides were stained with the Wright-Giemsa stain, and differential cell count was performed to determine the cell composition of the BAL fluid.

The dilution factor for BAL fluid and the volume of epithelial lining fluid (ELF) were calculated for each animal based on the urea concentration in serum and BAL fluid (Rennard et al., 1986).

**Determination of satratoxin G.** Sratatoxin G toxicity of spore extracts was determined using the luciferase translation inhibition test (Yike et al., 1999). Sratatoxin G content in BAL fluid was determined by an ELISA assay according to Chung et al. (2003), using reagents kindly provided by Dr. J. Pestka, University of Michigan.

**Proteolytic and hemolytic activity of spore extracts.** Fungal spores were suspended in a solution containing 2 mM Tris/HCl pH 7.4, 100 mM KCl, 1.5% polyvinylpyrrolidone and disrupted in a mini-bead beater with acid-washed glass beads. Cell breakage was evaluated microscopically. The suspensions were centrifuged for 20 min at 17,000 × g, and supernatants were collected. Enzymatic activity was measured using 2–5 μg protein of spore extracts and an EnzCheck® kit from Molecular Probes (Eugene, OR) with fluorescein-labeled gelatin as substrate. The activity was expressed as arbitrary fluorescence units per 10^6 spores. Hemolytic activity of spore extracts was assessed qualitatively by placing drops of extracts on sheep blood agar and incubating at 37°C for up to 72 h.

**Stachylysin assay.** Stachylysin in the BAL fluid was determined by ELISA using an affinity purified antibody directed against this protein as described previously (Van Emon et al., 2003).

**Electrophoretic analysis of bronchoalveolar lavage fluid (BAL) protein.** BAL fluid and serum samples (~15 μg/lane) were subjected to SDS-PAGE using 4–20% gradient gels from Bio-Rad (Hercules, CA) under reducing conditions. The gels were stained with Coomassie Blue R-250 (Bio-Rad, Hercules, CA).

**Cytokine assay.** The cytokines were assayed by ELISA using kits from R&D Systems (Minneapolis, MN) containing affinity purified antibodies to rat cytokines. The limits of detection for both IL-1β and TNF-α were 5.0 pg/ml.

**Protein assay.** Protein was measured according to the method of Bradford using the reagents from Bio-Rad (Hercules, CA).

**Statistical analysis.** All data were checked for normality and analyzed by one-factor (treatment) or two-way (factors: treatment and time) ANOVA using Tukey’s test for all pairwise multiple comparisons and Dunnett’s test for comparisons against the control group. While the weight and total BAL protein data followed normal distribution, other data required a logarithmic transformation. For morphometry data that were not normally distributed even after logarithmic transformation, nonparametric analysis of variance was performed. All statistical tests were performed using Sigmaplot version 2.03 (Jandel Scientific, San Rafael, CA), and the results were expressed as mean ± SEM and considered statistically significant at p < 0.05 probability level.

### RESULTS

#### Characterization of Spore Preparations

Intact, autoclaved, and ethanol-extracted spores were characterized with respect to their trichothecene toxicity/content, proteolytic and hemolytic activity, and viability. As shown in Table 1, intact spores collected from dry wall cultures are highly viable and contain trichothecenes levels equivalent to about 0.7 pg of satratoxin G per spore. The level of proteolytic activity in spore extracts obtained through spore breakage was equal to 0.41 arbitrary units (fluorescence) per million spores. Autoclaved spores fully retain their trichothecene toxicity, while their proteolytic and hemolytic activity and ability to germinate is completely eliminated. Ethanol-extracted spores are devoid of trichothecenes, contain no proteinase or hemo-lysin activity, and are nonviable.

#### Animal Growth and Survival

Treatment of 7-day-old rats with intact, viable spores at 1 × 10^7 spores/g BW results in 14% mortality. Hemorrhagic lungs were observed in animals recovered for autopsy. There were no deaths in the experimental animals treated with autoclaved and ethanol-extracted spores or in the control group exposed to PBS.

The growth of surviving animals exposed to intact spores was significantly reduced at 24, 36, 48, and 72 h after exposure (p < 0.05; Fig. 1). At 48 h these animals reached their original weight and continued to grow, although they remained smaller than controls. The growth of animals treated with autoclaved spores was significantly different from controls only at 24, 36, and 72 h. Statistically significant differences (p < 0.05) between the animals treated with intact and autoclaved spores were noted at 36, 48, and 60 h. There was no discernable impact of ethanol-extracted spores on the growth of infant animals.

#### Inflammatory Response in the Lung

**Histological analysis.** Increased numbers of inflammatory cells and the presence of spore-containing granulomas were observed in all three experimental groups in contrast to PBS-treated controls (Figs. 2A–2C, 72 h after exposure). Those effects appear to be the most severe in the animals exposed to...
intact spores and were progressively less in the groups exposed to autoclaved and extracted spores. These qualitative observations were confirmed by a quantitative morphometric analysis showing a rapid reduction in alveolar space during the first 12 h after exposure in animals treated with all spore preparations compared to PBS-treated controls ($p < 0.05$, Fig. 3). The largest effects were observed in pups treated with intact spores, where the alveolar space continued to decrease during the next 12 h, reaching 42.1% at 24 h compared to 56.8% for the animals treated with autoclaved spores, 51.1% for the animals treated with ethanol-extracted spores and 60.6% for PBS-treated controls ($p = 0.001$ for comparison of every spore preparation and PBS-treated animals). While the effect of ethanol-extracted spores appeared to be larger than that of autoclaved spores, there were no statistically significant differences at any time point noted between the animals treated with these two spore preparations (Fig. 3).

Bronchoalveolar lavage analysis. The cellular composition of bronchoalveolar lavage fluid was affected in rat pups exposed to fungal spores. The level of erythrocytes in the BAL fluid of the animals treated with intact spores was slightly elevated at 6 h post treatment and reached a sharp peak at 24 h ($p = 0.002$ compared to PBS-treated control animals), similarly to the peak of neutrophils and remained a little higher than controls for another 24 h (Fig. 4). No significant increases were observed in autoclaved, ethanol-extracted and PBS treatment groups.

For intact spores, the level of macrophages rose after 6 h and reached its maximum at 60 h following exposure ($p = 0.025$ compared to PBS-treated controls). A similar increase ($p = 0.025$ compared to PBS-treated controls) seen during the first 24 h in the animals treated with autoclaved spores was followed by a decline and was not statistically different from controls after 36 h. The initial small differences between the ethanol-extracted spores group and PBS-treated control animals were not statistically significant.
As shown in Figure 4C, the numbers of neutrophils were highly elevated at 16 h following exposure to intact spores of *S. chartarum* reaching the highest level at 24 h (\( p = 0.002 \) compared to PBS-treated control animals). This was followed by a fall in the number of neutrophils at 36 h and another increase at 48 h. After 60 h the number of neutrophils recovered in the BAL fluid started to decrease. Autoclaved spores elicited a much smaller neutrophil response observed between 16 and 60 h after exposure (\( p = 0.002 \) compared to PBS-treated animals). This response was even weaker but still statistically significant in the animals exposed to ethanol-extracted spores (\( p \leq 0.002 \) compared to control animals). The levels of neutrophils in PBS-treated control group were low (\( 4 \times 10^4 \) cells/ml of epithelial lining fluid) and did not increase with time.

Increases in the levels of lymphocytes were small and highly variable in all treated animals until after 48 h, when they appeared to raise sharply in the animals treated with intact spores (Fig. 4D).

Proinflammatory cytokines (TNF-\( \alpha \) and IL-1\( \beta \)) were detected in the BAL fluid of animals treated with different preparations of the spores of *S. chartarum* in contrast to PBS-treated group, where no cytokines could be detected. The highest increases were seen with intact spores, followed by autoclaved and ethanol-extracted spores. The concentrations of IL-1\( \beta \) reached the highest level at 24 h post-exposure (Fig. 5A) in all of the experimental groups (\( p \leq 0.015 \) compared to control animals).
In rat pups treated with ethanol-extracted spores, the increases in the level of IL-1β were relatively small, and the 24-h maximum concentration was not significantly different from those of 16 and 48 h. TNF-α reached its highest concentration at 16 h post-exposure (Fig. 5B, \( p = 0.002 \)) in animals treated with intact spores and then decreased rapidly. Exposure to autoclaved spores led to a smaller induction of this cytokine with a peak at 24 h (\( p = 0.024 \)). Extracted spores elicited a much weaker response, with the highest concentration detected at 48 h post-exposure (not statistically significant compared to control group).

Total protein in the BAL fluid of animals exposed to the spores of \( S. \) chartarum was rapidly elevated in all three of the experimental groups and was significantly different \(( p < 0.05)\) from that of controls at 3 and 6 h after exposure. Maximal levels were observed at 3 h in rat pups exposed to the autoclaved and extracted spores (Fig. 6), while in the intact-spores-treated animals, the protein level continued to rise and remained high to 16 h. Similarly to other responses, concentrations of the total BAL fluid protein were the highest in animals exposed to intact spores, followed by autoclaved and extracted-spore preparations. Total protein in the BAL fluid of PBS-treated animals also increased during the first 16 h after treatment. Serum albumin remained the main protein component of the BAL fluid over 72 h in the animals treated with intact and autoclaved spores, based on the comparison of the electrophoretic profile of BALF and serum samples (data not shown).

Release of Sattratoxin G and Stachylysin

The release of trichothecene mycotoxins and stachylysin was studied in separate experiments. A higher dose of 400,000 sp/g BW (268 µg sattratoxin G per kg body weight) was instilled to facilitate sattratoxin detection, and bronchoalveolar lavage was performed at 0, 15, 45, 60 min and at 2, 3, 4, 5, 6, 16, and 24 h. To measure stachylysin, infant rats were exposed to 100,000 sp/g BW of \( S. \) chartarum strain JS58-06 (no sattratoxin, high stachylysin activity) and subjected to bronchoalveolar lavage on day 1, 2, 3, 4, 7, 21, and 28. The time-course data of sattratoxin G and fungal protein release from the spores of \( S. \) chartarum are shown in Figure 7. Following intratracheal exposure to the intact spores of the JS58-17 isolate, sattratoxin G was detected in the BAL fluid at time 0, and its concentration rapidly declined in the first 15 min after exposure and remained very low in the course of 24 h (Fig. 7A). In contrast, the concentration of stachylysin in the BAL fluid reached its maximum on day 4 post-exposure, declined slowly, and could still be detected at 28 days (Fig. 7B).

DISCUSSION

Overall, the magnitude and time course of pulmonary responses to the spores of \( S. \) chartarum are similar to those reported by Rao et al. (2000a,b) in young adult rats, with many of the inflammatory parameters reaching their maximum at
24 h post-exposure. Similar effects were seen in other animal models of lung injury, i.e., those caused by exposure to endotoxin (Moffat et al., 2002), while Flemming et al. (2004) and Rand et al. (2003) observed somewhat different time courses in their murine models of exposure to S. chartarum (see below).

Intact, viable spores of S. chartarum had the strongest effect on survival and growth of the animals and on all of the inflammatory indices including erythrocytes, leukocytes, proinflammatory cytokines, and alveolar space. Those effects were progressively reduced in the animals exposed to autoclaved and ethanol-extracted spores. The time course of the responses observed in this study confirms reports of Rao et al. (2000a,b) and our earlier findings (Yike et al., 2001) indicating that the first 24–48 h after exposure corresponds to the period of acute toxicity, which is then followed by partial recovery and sustained inflammation with developing granulomas. All exposure-related deaths occurred within 48 h. While ethanol-extracted spores had no effect on survival and growth of exposed rat pups, viable spores significantly affected the weight gain and resulted in 14% mortality. Autoclaved spores did not cause deaths but also significantly reduced the growth rate during the first 24 h. This reduction was followed by a recovery that was much faster compared to the animals exposed to intact spores. Thus, the effects of heat-stable mycotoxins without proteins were more short-lived. It has been postulated (Flemming et al., 2004) that depressed weight change in mice may be related to proinflammatory cytokines induced by S. chartarum that may lead to appetite suppression (Kuby, 1997). Both the levels and the time course of cytokine release in the BAL fluid agree with such interpretation.

The decreases of alveolar space in the animals exposed to different spore preparations paralleled other inflammatory parameters in that differences between the control animals and those exposed to the intact, autoclaved, and ethanol-extracted spores were highly significant, although there was no clear distinction between the effects of autoclaved and extracted spores. This may be related to technical difficulties in attaining sufficiently uniform levels of lung inflation. We have observed that the lungs of infant rats with large areas of inflammation tend to remain underinflated due to airway obstruction, which may undermine the accuracy of morphometric analysis, especially when small changes are considered (i.e., in case of extracted vs. autoclaved spores).

The differences between the effects of autoclaved and intact spores indicate that, in addition to trichothecenes, other components of the spores of S. chartarum are also involved in the lung inflammation and injury. While the autoclaved spores retain at least the macrocyclic trichothecene secondary metabolites compared to the intact spores, their proteinaceous components are clearly inactivated. Comparing the preparations of autoclaved spores to intact, untreated spores allows for differentiation of the effects of trichothecenes (and other heat-stable mycotoxins) from that of proteins and/or other components that are inactivated during autoclaving. Such comparison indicates that those components of the spores are as important if not crucial in the pathophysiology of spore inhalation. The residual effects observed with ethanol-extracted spores are likely to result from the response to β-glucan. However, assuming that 1 → 3, β-D-glucan is not significantly affected by the autoclaving and extraction procedures used, this spore component appears to be a minor contributor to the pathophysiology as suggested by Korpi et al. (2003), who observed only minor irritation when exposing mice to that cell wall polymer.

Flemming et al. (2004) observed that high trichothecene producing S. chartarum isolate JS58-17 evoked relatively fast responses in mice, generally within 24 h followed by a decline, while the responses stimulated by the nontoxic isolate JS58-06 increased throughout a 96-h period. These differences suggest that trichothecenes may be the main factor responsible for the early responses that peaked at 24 h. Later effects may depend more on the release of other fungal factors (i.e., proteases and hemolysin). Such interpretation is in agreement with earlier studies of Creasia et al. (1987) and more recent findings of
Rand et al. (2002), who were unable to observe inflammatory lesions in the lungs of animals exposed to pure trichothecenes T-2 toxin and isosatratoxin-F. It has been suggested (Pang et al., 1987) that T-2 toxin is rapidly absorbed from the lung, metabolized, and excreted. Demonstration that satratoxin G could only be detected in the BAL fluid of infant rats immediately following exposure to the spores further supports this view. Unpublished observations by Jarvis (personal communication) also suggest that S. chartarum, similar to other fungi (Demain, 1981), releases a major portion of the trichothecene toxin from the surface of the spores. Immunohistochemical localization of satratoxin within the spores of S. chartarum found it to be primarily along the outer plasma-lemma surface and in the inner wall layer (Rand et al., 2004) consistent with rapid release from the spores.

In contrast to the rapid release and absorption of satratoxin, stachylysin, a hemolytic protein from S. chartarum (Vesper et al., 2001), was slowly released from the spores into the lung, reaching its maximal concentration in the BAL fluid 4 days after exposure, and could still be detected at 28 days. We have previously reported similar results (Gregory et al., 2003) showing that more stachylysin can be labeled by immunohistochemistry at 72 h than at 24 h post-exposure.

Proinflammatory cytokines, neutrophils, and BAL fluid protein appear to be the most sensitive indicators of inflammation, because they either cannot be detected in PBS control animals (cytokines) or are detected at very low concentrations (neutrophils, protein) and increase rapidly after exposure to the spores of S. chartarum. The second peak of neutrophils in the BALf from animals treated with intact spores may be related to another influx of these cells in response to continued proteolytic injury from host proteinases in addition to fungal proteinases. The neutrophil decline between these peaks may reflect apoptotic death and clearing by macrophages. Trichothecenes including satratoxins have been shown to cause apoptosis of leukocytes (Yang et al., 2000). Proinflammatory cytokines including TNF-α have been implicated in the lethality of other toxins, i.e., Shiga toxins (Sasaki et al., 2002).

Induction of proinflammatory cytokines measured in the BAL fluid of mice exposed to S. chartarum (Flemming et al., 2004) exhibited somewhat different time profiles, with the maximum response for TNF-α seen at 3 h. Although the exact reason for these differences remains unclear, it may stem from the apparent increased susceptibility of mice seen in different dose-related responses. This is not surprising in light of the reports of different susceptibility to S. chartarum observed within different strains of mice (Rosenblum et al., 2002). Higher susceptibility of mice compared to rats to other toxic inhalants has also been reported (Csanday et al., 2003).

It has been our working hypothesis that early effects such as growth impairment and significant increases in neutrophils, erythrocytes, and proinflammatory cytokines are mediated primarily by trichothecene mycotoxins, while the sustained lower level of inflammation could be attributed to the more slowly released fungal proteins. The rapid increase in total protein appears to support such interpretation. However, significant differences in inflammatory indices between intact and autoclaved spores, seen shortly after exposure, point to the involvement of fungal proteins also in the early events even before those proteins reach their peak concentrations and, perhaps, their synergistic action with trichothecenes. There seems to be no clear difference in timing between the cytotoxic effects of trichothecenes and the proinflammatory action of other fungal-derived agents as was suggested by others (Korpi et al., 2002; Nielsen et al., 2002; Murtoniemi et al., 2001).

While the increased responses seen with intact, viable spores compared to autoclaved spores tend to underscore the involvement of fungal proteins, at least some of those responses might be related to an increase in fungal mass (i.e., higher concentrations of mycotoxins, including trichothecenes, proteins, and other metabolites) that might take place right before and during germination of the spores. Although no germination or formation of fungal hyphae was noted under the experimental conditions of this study, we have previously observed germination and limited persistence of Stachybotrys chartarum in 4-day-old rat pups (Yike et al., 2003). Even if there is no germination in the lungs of 7-day-old pups, intense synthesis and release of metabolites by highly viable spores is possible and may, at least partially, account for what could be otherwise interpreted as synergistic effects of mycotoxins and fungal proteins. We have observed large increases in the concentration of total protein secreted from the spores during incubation in vitro, while the concentration of satratoxin G remained constant (unpublished). Thus, it appears that the spores may not synthesize satratoxin G within the first 72 h but apparently can produce large quantities of proteins.

The possibility of involvement of fungal proteins in the pathophysiology of spore inhalation sheds new light on the clinical significance of those isolates of S. chartarum that do not produce macrocyclic trichothecenes but have higher hemolytic and proteolytic activity than high trichothecene producers (Yike and Dearborn, 2004). In addition, the synergistic action of mycotoxins, proteins, and other fungal agents may explain the adverse pulmonary effects of the relatively low spore concentrations found in indoor air.

ACKNOWLEDGMENTS
The authors thank Jody Gower and Carrie McCaughtry for excellent technical assistance. This work was supported by NIEHS grant ES009742.

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