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Reduction of Pulmonary Toxicity of *Stachybotrys chartarum* Spores by Methanol Extraction of Mycotoxins

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The fungus *Stachybotrys chartarum* has been implicated in cases of nonspecific indoor air quality complaints in adults and in cases of pulmonary hemorrhaging in infants. The effects that have been described have been attributed to mycotoxins. Previous dose-effect studies focused on exposure to a single mycotoxin in a solvent, a strategy which is unlikely to accurately characterize the effects of inhaled spores. In this study we examined the role of mycotoxins in the pulmonary effects caused by *S. chartarum* spores and the dose dependency of these effects. *S. chartarum* spores were extracted in methanol to reduce the mycotoxin content of the spores. Then either untreated (toxin-containing) or methanol-extracted *S. chartarum* spores were intratracheally instilled into male 10-week-old Charles River-Dawley rats. After 24 h, the lungs were lavaged, and the bronchoalveolar lavage fluid was analyzed to determine differences in lactic dehydrogenase, albumin, hemoglobin, myeloperoxidase, and leukocyte differential counts. Weight change was also monitored. Our data show that methanol extraction dramatically reduced the toxicity of *S. chartarum* spores. No statistically significant effects were observed in the bronchoalveolar lavage fluids of the animals that were treated with methanol-extracted spores at any dose. Conversely, dose-dependent effects of the toxin-containing spores were observed when we examined the lactic dehydrogenase, albumin, and hemoglobin concentrations, the polymorphonuclear leukocyte counts, and weight loss. Our findings show that a single, intense exposure to toxin-containing *S. chartarum* spores results in pulmonary inflammation and injury in a dose-dependent manner. Importantly, the effects are related to methanol-soluble toxins in the spores.

Acute exposure to *Stachybotrys chartarum* spores results in severe pulmonary injury in animal models (15, 16, 21). Although several outbreaks of illness in humans have been attributed to respiratory exposure to *S. chartarum*, the causal link between fungal contamination in the indoor environment and adverse pulmonary effects has yet to be firmly established (4–6, 9, 13, 25).

The majority of the data regarding dose-response relationships between mycotoxin exposure and health effects are 50% lethal dose data resulting from ingestion or injection exposure in animals (10, 26). There are no data on the effects of low doses and the effects of inhaled mycotoxins. One limiting factor in studies is the ability to detect the pulmonary effects of low doses, since such doses may not result in mortality or detectable histological changes. In addition, *S. chartarum* can concurrently produce several different mycotoxins, each of which has different effects. These toxins may act synergistically, and particle association may contribute to toxicity (1, 2, 14, 23).

Bronchoalveolar lavage (BAL) has been used to detect adverse pulmonary effects resulting from indoor exposure to fungi (17). The indicators measured with BAL can be used to quantitatively evaluate pulmonary edema (albumin), cytotoxicity (lactic dehydrogenase [LDH]), polymorphonuclear leukocyte (PMN) activation (myeloperoxidase [MPO]), and pulmonary hemorrhaging (hemoglobin). Differential leukocyte enumerations for alveolar macrophages, PMNs, lymphocytes, and eosinophils are used to assess cellular inflammatory and immunological responses. In the study described here, we used BAL to investigate the dose-effect relationship between exposure to *S. chartarum* spores and pulmonary effects. To differentiate between mycotoxin-induced injury and the effects of other fungal spore components (e.g., allergens, β-glucans), we also tested *S. chartarum* spores that had been extracted with methanol to reduce the mycotoxin concentration in them.

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MATERIALS AND METHODS

Fungal strains and spore suspensions. Eight strains of *S. chartarum* that were isolated from a Southern California residence were screened for toxin production by using a modified brine shrimp lethality assay as described by Eppley (8). A toxin-producing strain was selected for the experiments. The strain of *S. chartarum* used was maintained on potato dextrose agar slants at 15°C. Spores were vacuumed from the surfaces of 14-day agar cultures by using a modified filter cassette with a 37-mm-diameter, 0.4-μm-pore-size polycarbonate membrane filter (Poretics Corp., Livermore, Calif.). The spores were then suspended in 0.9% saline to concentrations of 2×10^6, 4×10^6, 1×10^7, and 2×10^7 spores per ml. A reduced-toxin *S. chartarum* spore suspension was prepared from the same spore harvest by performing agitated extraction with 100% methanol (Sigma, Lenexa, Kans.) for 30 min. After centrifugation at $350 \times g$ for 10 min, the spore pellet was collected, washed in fresh methanol, centrifuged, washed in saline, and then suspended in fresh saline to concentrations of 2×10^6, 4×10^6, 1×10^7, and 2×10^7 spores per ml. The concentrations and nature of the spore suspensions were evaluated by light microscopy at a magnification of ×200 in a hemocytometer chamber. Minor hyphal fragments and negligible spore clumping were observed, and the majority of the particles were ovoid spores with a mean size of 6.1 by 8.8 μm.

BAL procedures. Male 10-week-old Charles River-Dawley rats were supplied by Charles River Breeding Corporation. The average initial weight of the animals was 332 ± 26.6 g. The rats were housed in isolation at 25°C for at least a 1-week acclimation period and were fed Purina Rat Chow and water ad libitum.

The spore suspensions were delivered into the lungs of the animals by intratracheal instillation. The rats were anesthetized by inhalation of 5% halothane gas and then were placed on a slanted board. A freshly prepared spore suspension or a saline carrier control was instilled into the lungs of each animal with a 1-ml disposable tuberculin syringe attached to a 3.5-in., 19-gauge, bent, blunt-tip needle inserted between the vocal folds. Tracheal insertion was verified by detection of the cartilaginous rings by the needle. The volume of the carrier control (saline) or the spore suspension instilled was 150 μl per 100 g of body weight. The animal remained in the slanted position until it recovered minimally, approximately 1 min. Then it was placed on its back in a cage, where it regained consciousness within a few minutes. After instillation, the animals were kept in the laboratory under preinstillation housing and maintenance conditions.

After 24 h, the rats were reweighed, injected with 1 ml of sodium pentobar-

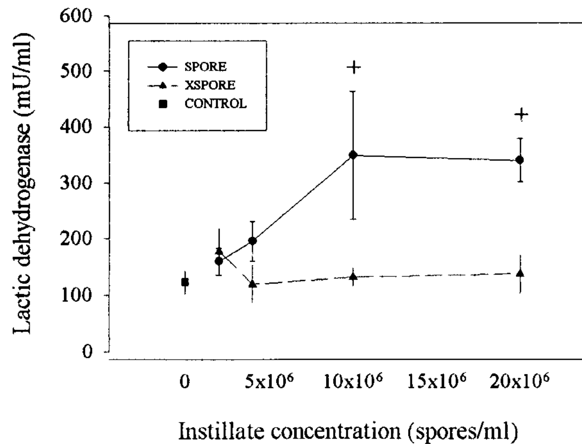

FIG. 1. Mean concentrations of LDH in BAL fluid 24 h after *S. chartarum* spore instillation. The error bars indicate standard errors. A plus sign indicates that a P value is <0.05 for within-dose comparisons between animals instilled with untreated *S. chartarum* spores (SPORE) and animals instilled with methanol-extracted *S. chartarum* spores (XSPORE), with the caveat that the multiple-comparison F statistic of the ANCOVA is borderline significant ($P = 0.12$).

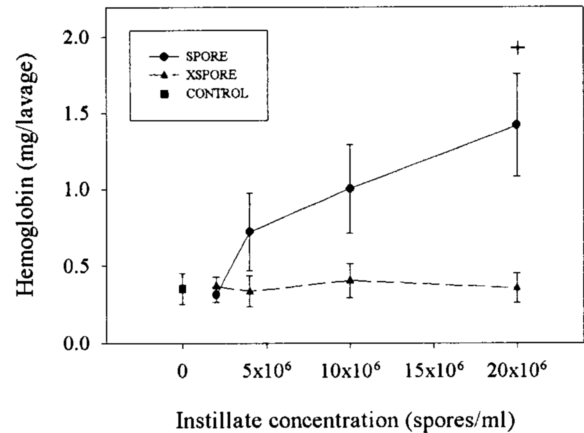

FIG. 2. Mean concentrations of hemoglobin in BAL fluid 24 h after *S. chartarum* spore instillation. The error bars indicate standard errors. A plus sign indicates that the P value is <0.05 for within-dose comparisons between animals instilled with untreated *S. chartarum* spores (SPORE) and animals instilled with methanol-extracted *S. chartarum* spores (XSPORE), with the caveat that the multiple-comparison F statistic of the ANCOVA is borderline significant ($P = 0.18$).

bital, and then exsanguinated by cutting the abdominal aorta. The trachea was exposed and cannulated with an 18-gauge, blunt-tip needle fitted with a 1-in., flared, 19-gauge, polypropylene tube and a 5-ml disposable syringe. The lungs were lavaged in situ 12 times by injecting 3 ml of phosphate-buffered saline into them and then massaging the ribcage while the lavage fluid was aspirated. The first two lavage aliquots were combined (approximately 5 ml) and centrifuged under refrigerated conditions at 350 $\times$ *g* for 10 min. The supernatant was reserved for use in LDH, MPO, and albumin analyses. The resulting cell pellet was combined with the cell pellet obtained from the next 10 lavage aliquots and used for the cytospin and hemoglobin analyses.

Analysis of BAL fluid. The total leukocyte count in the lavage fluid was determined in a hemocytometer chamber at a magnification of $\times$100. Leukocytes were identified on the basis of size and granularity. After a cytospin (72 $\times$ *g*, 5 min) onto a microscope slide, the deposited cells were fixed, stained, and mounted in Permount. Differential counts for macrophages, PMNs, eosinophils, and lymphocytes were determined at a magnification of $\times$200 by light microscopy.

The supernatant reserved from the first two lavage aliquots was recentrifuged under refrigerated conditions at 14,500 $\times$ *g* for 30 min, and the resultant supernatant was analyzed to determine LDH, MPO, and albumin concentrations by spectrophotometry. The combined cell pellet obtained from all 12 lavage aliquots was analyzed for hemoglobin. The supernatants of some of the lavage aliquots from the animals that received the highest dose were red tinged (instead of clear). This indicated that some erythrocytes had been lysed; therefore, we may have underestimated the total hemoglobin concentration in some of the BAL samples by analyzing only the cell pellet. Biochemical analyses with a spectrophotometer were performed as described by Beck et al. (3).

Statistical analyses. Overall, 42 animals were instilled with an untreated *S. chartarum* spore suspension (SPORE animals), a methanol-extracted spore suspension (XSPORE animals), or a carrier control (CONTROL animals). For each instillate type, at least four animals were studied with each instillate concentration (2×10^6, 4×10^6, 1×10^7, 2×10^7 spores/ml of instillate). All indicators (concentrations of LDH, hemoglobin, albumin, and MPO; total number of macrophages; total number of PMNs; total number of eosinophils; total number of lymphocytes; and percent weight change) were examined for each animal.

Statistical analyses were performed by using SAS statistical software (22). Analyses to determine the trends (SAS PROC MULTTEST) for the BAL indicators and weight change were performed for each instillate type (untreated *S. chartarum* spores and methanol-extracted *S. chartarum* spores). If a trend was significant for an indicator, we used an analysis of variance (ANOVA) with Dunnett's test (SAS PROC GLM) to determine which responses to doses were significantly different from the carrier control animal responses. Some of the BAL indicators were transformed to obtain an approximate Gaussian distribution of the residuals.

To determine if the BAL indicators for untreated spores and methanol-extracted spores were different, we used an analysis of covariance (ANCOVA) to compare the BAL indicators for animals instilled with untreated *S. chartarum* spores, animals instilled with methanol-extracted *S. chartarum* spores, and animals instilled with the saline carrier control (SAS PROC GLM). We constructed a linear model in which the main effects were instillate type, dose, and the interaction of instillate type and dose. To account for multiple comparisons, we examined only within-dose comparisons between untreated *S. chartarum* spores and methanol-extracted *S. chartarum* spores when the F statistic for the interaction effect was significant. The differences between least-square means derived from the model were used to determine statistical significance. The critical value used was $P < 0.05$.

RESULTS

Methanol-extracted *S. chartarum* spores. No linear trends were observed for most of the BAL indicators or for weight change for animals instilled with methanol-extracted spores (XSPORE animals); the only exception was MPO ($P = 0.02$ [data not shown]). Within the data set, 70% of the MPO measurements were below the limit of detection, resulting in small variances which may explain the apparent positive trend. The LDH (Fig. 1), hemoglobin (Fig. 2), and albumin (Fig. 3) concentrations; the macrophage, PMN, lymphocyte, and eosinophil counts (Fig. 4); and the weight change percentages for the XSPORE animals (Fig. 5) were not significantly different than

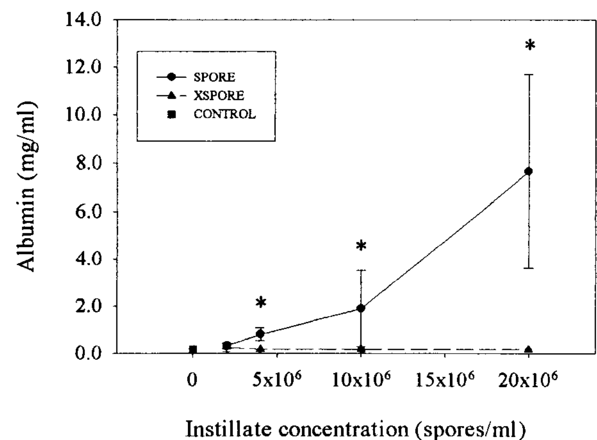

FIG. 3. Mean concentrations of albumin in BAL fluid 24 h after *S. chartarum* spore instillation. The error bars indicate standard errors. Animals instilled with untreated *S. chartarum* spores (SPORE) were compared to animals instilled with methanol-extracted *S. chartarum* spores (XSPORE). An asterisk indicates that the P value is <0.05.

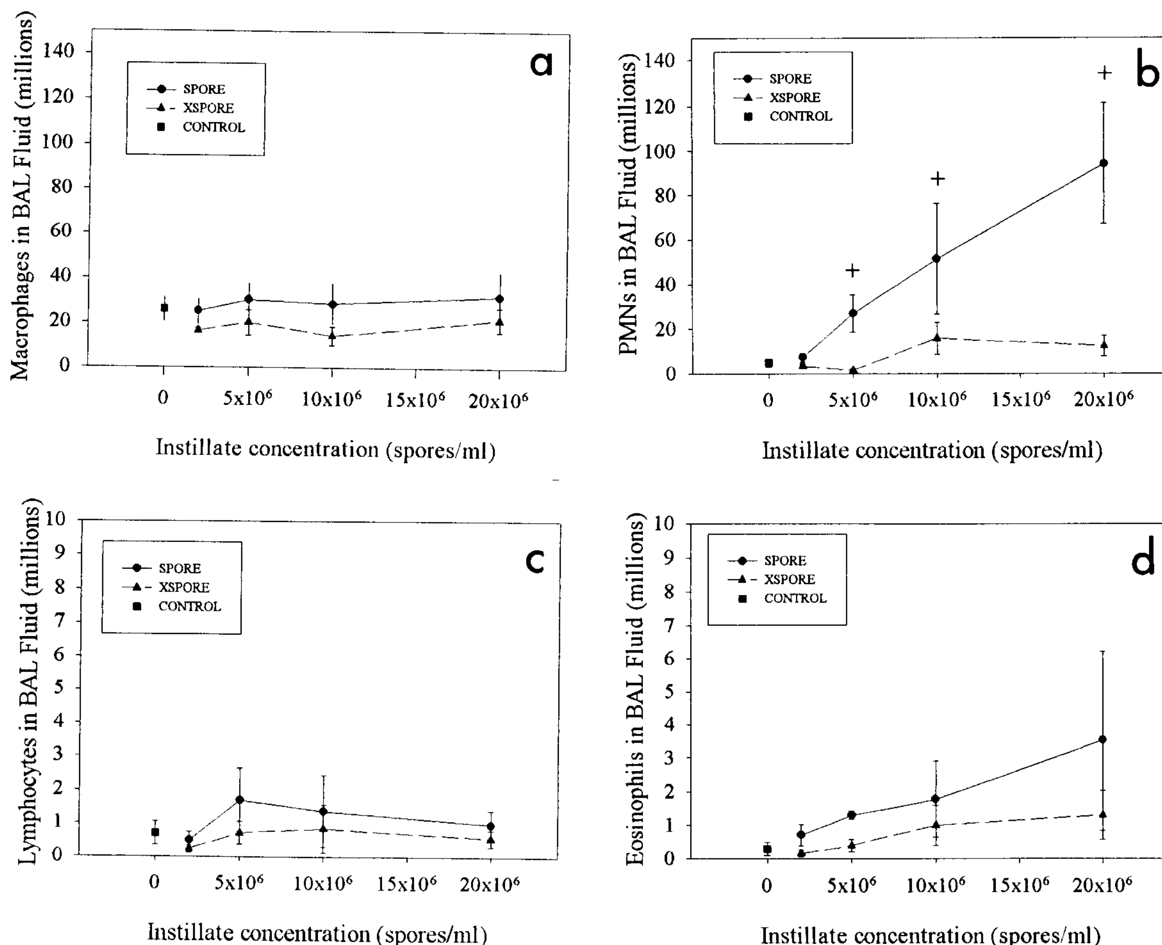

FIG. 4. Mean macrophage (a), PMN (b), lymphocyte (c), and eosinophil (d) counts in BAL fluid 24 h after *S. chartarum* spore instillation. The error bars indicate standard errors. A plus sign indicates that the *P* value is <0.05 for within-dose comparisons between animals instilled with untreated *S. chartarum* spores (SPORE) and animals instilled with methanol-extracted spores (XSPORE) with the caveat that the multiple-comparison ANCOVA F statistic for total PMN counts is borderline significant ($P = 0.09$).

the values obtained for the CONTROL animals at the experimental doses used.

Untreated *S. chartarum* spores. (i) Physiological effects: weight change. As the untreated *S. chartarum* spore instillate concentration increased (SPORE animals), the animals lost more weight ($P = 0.0001$) (Fig. 5). There were significant differences compared to the weights of the CONTROL (Table 1) and XSPORE (Fig. 5) animals at the two highest instillate concentrations.

(ii) Pulmonary injury and inflammation. The LDH ($P = 0.003$) and hemoglobin ($P = 0.005$) concentrations increased linearly as the untreated *S. chartarum* spore instillate concentration increased (Fig. 1 and 2, respectively). Compared to the values for the CONTROL animals, the concentrations of LDH in the BAL fluid were significantly different at the two highest doses and the concentration of hemoglobin was significantly different at the highest instillate dose (Table 1). When the SPORE and XSPORE animal instillates were compared, the hemoglobin or LDH concentrations in the BAL fluid were not significantly different ($P = 0.18$ and $P = 0.12$, respectively). However, with the caveat that the multiple-comparison F statistics were not significant, the within-dose differences between the SPORE and XSPORE animals exhibited the same pattern

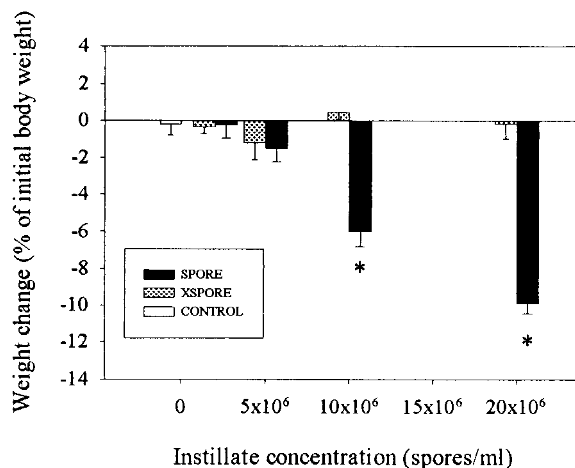

FIG. 5. Mean percent weight change 24 h after instillation. The error bars indicate standard errors. Animals instilled with untreated *S. chartarum* spores (SPORE) were compared to animals instilled with methanol-extracted *S. chartarum* spores (XSPORE). An asterisk indicates that the *P* value is <0.05.

TABLE 1. *P* values from an ANOVA model comparison: animals instilled with untreated *S. chartarum* spores (SPORE animals) versus animals treated with a saline carrier (CONTROL animals)[a]

Indicator	ANOVA F statistic (*P* value)[b]	*P* value for:			
		SPORE (2×10^6 spores/ml) vs CONTROL	SPORE (4×10^6 spores/ml) vs CONTROL	SPORE (10×10^6 spores/ml) vs CONTROL	SPORE (20×10^6 spores/ml) vs CONTROL
% Weight change[c]	48.61 (**0.0001**)	1.00	0.49	**0.0001**	**0.0001**
LDH[c]	5.24 (**0.005**)	0.87	0.49	**0.014**	**0.003**
Hemoglobin[c]	3.79 (**0.018**)	1.00	0.49	0.14	**0.001**
Albumin[c]	29.02 (**0.0001**)	0.34	**0.004**	**0.0003**	**0.0001**
PMNs[c]	9.88 (**0.0002**)	0.60	**0.01**	**0.003**	**0.0001**
MPO	3.61 (**0.022**)	0.95	**0.05**	**0.02**	0.87
Macrophages	0.13 (0.97)				
Lymphocytes	0.98 (0.45)				
Eosinophils	1.96 (0.16)				

[a] *P* values for dose comparisons are given only if the F statistic of the model was significant. *P* values that are ≤0.05 are indicated by boldface type.
[b] ANOVA performed with Dunnett's test.
[c] There was a significant linear trend for unadulterated *S. chartarum* spores.

that the SPORE and CONTROL animal differences exhibited (Table 1).

The albumin concentration (Fig. 3) and the total PMN count (Fig. 4b) increased significantly as the SPORE dose increased ($P = 0.0001$ for both indicators). Linear trends were not observed with the MPO concentration or with the other leukocyte counts (macrophages, lymphocytes, and eosinophils). The albumin concentrations and the total PMN counts in the BAL fluid from SPORE animals were significantly different from the values for the CONTROL animals (Table 1) and the XSPORE animals at instillate concentrations equal to or greater than 4×10^6 spores/ml.

DISCUSSION

Mycotoxin-specific effects. In vitro studies have shown that nontoxic, noninfective species of fungi can modulate production of cytokines and reactive oxygen species, which in turn can elicit pulmonary inflammation (19, 24). Mycotoxins are highly soluble in organic solvents and slightly soluble in physiological fluids (11, 18). We extracted *S. chartarum* spores in methanol to reduce their mycotoxin content. The pulmonary responses of animals instilled with methanol-extracted spores were similar to the pulmonary responses of CONTROL animals and significantly less than the pulmonary responses of animals instilled with untreated *S. chartarum* spores. The lack of a linear trend for any of the indicators measured (except MPO) in animals treated with methanol-extracted spores demonstrated that the methanol-extractable fraction played a significant role in pulmonary inflammation.

Some glucans, extracellular polysaccharides, lipids, and allergens, which can also cause inflammation, may also have been extracted or altered by the methanol. In addition, the methanol treatment sterilized the spores. Thus, the lack of effects associated with the methanol-extracted *S. chartarum* spores cannot be attributed solely to the absence of mycotoxins. However, previous research in our laboratory has shown that non-mycotoxin-containing fungal spores (i.e., *Penicillium chrysogenum* and *Cladosporium sphaerospermum*) have dose-effect relationships for the BAL indicators similar to the dose-effect relationships of the methanol-extracted *S. chartarum* spores (J. D. Brain, T. Donaghey, C. Y. Rao, and H. A. Burge, unpublished data). They are also less toxic than nonextracted *S. chartarum* spores. Moreover, *S. chartarum* spores that exhibit low toxicity (as measured by cell culture assays) have also been shown to have diminished effects on pulmonary tissue (16).

Dose-related toxic effects of untreated *S. chartarum* spores. (i) Physiological effects. Overt physiological effects of acute pulmonary exposure to *S. chartarum* were reflected by dose-dependent decreases in body weight. Within 24 h after instillation of untreated spores, the animals that received the highest doses lost up to 13% of their body weight, while CONTROL animals generally remained the same weight. The acute weight loss suggests that consumption of food and water decreased, which could have resulted from general malaise related to local effects or from systemic toxicity. In some human case studies, systemic effects such as malaise, flulike symptoms, muscle aches, and headaches have been associated with postulated airborne exposure to *S. chartarum* spores (6, 7, 12, 13).

(ii) Pulmonary injury and inflammation. The increased levels of LDH, hemoglobin, albumin, and PMNs in the BAL fluid reflect the direct effects of *S. chartarum* spores and their constituents on lung tissue. In normal lungs, extracellular LDH (resulting from cytotoxicity and cell death) and hemoglobin (resulting from erythrocyte infiltration through capillary bed perforations) are not detectable in the lavage fluid. Both of these indicators are sensitive indicators of localized injury, a likely result if a large number of spores were deposited in a relatively small area of tissue. This effect was demonstrated by the significant changes in LDH and hemoglobin concentrations that were observed at the higher doses. Mucosal hemorrhaging and tissue necroses have been observed in animals that have ingested large amounts of *S. chartarum*-contaminated fodder (12, 20). Although trichothecenes are potent inhibitors of protein synthesis, the specific mechanisms of action that result in localized injury are not known.

An increase in the level of albumin, a serum protein that is abundant in BAL fluid, may be an early and sensitive indicator of widespread inflammation. Significant differences from CONTROL animals were apparent at lower spore instillate concentrations for albumin than for LDH and hemoglobin. Moreover, significant increases in albumin concentrations were observed as soon as 6 h after pulmonary exposure to *S. chartarum* spores (C. Y. Rao, H. A. Burge, and J. D. Brain, unpublished data).

The total leukocyte counts in BAL fluid increased, and PMNs were the major contributor to this increase. Total macrophages, lymphocytes, and eosinophils did not increase as the

instillate concentration increased. One day may not be long enough for such changes to occur. We have observed significant increases in macrophage counts 72 h after instillation (Rao et al., unpublished data).

It has been hypothesized that the presence of *S. chartarum* in occupied spaces is responsible for building-related adverse health effects, including pulmonary hemorrhaging. Mycotoxins in the spores are the presumed causative factor. Documenting such effects requires demonstrating that (i) *S. chartarum* spores in the environment contained toxins; (ii) the observed effect is related to the toxin content rather than to other spore components or other nonfungal factors; and (iii) sufficient toxin exposure occurs to achieve a toxic dose. We provide evidence that there is a dose-related association between an acute exposure to toxin-containing *S. chartarum* spores and measurable pulmonary responses. The consequences of low-level chronic exposure remain to be investigated, as does the relevance of the rodent data to human exposure.

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