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Comparison of Inflammatory and Cytotoxic Lung Responses in Mice after Intratracheal Exposure to Spores of Two Different *Stachybotrys chartarum* Strains

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***Stachybotrys chartarum* is an important toxigenic fungus that has been associated with respiratory disease onset in animals and humans. While it can be separated into macrocyclic trichothecene- and atranone-producing chemotypes based on secondary metabolite production, effects of spores of the two chemotypes on lungs are poorly understood. In this study we used bronchoalveolar lavage fluid (BALF) to investigate dose-response (30, 300, 3000 spores/g body weight [BW]) and time-course (3, 6, 24, 48, 96 h post instillation [PI]) relationships in mice to exposure of macrocyclic trichothecene- (JS 58-17) and atranone-producing (JS 58-06) *S. chartarum* strains, as well as *Cladosporium cladosporioides* spores. BALF total protein, albumin, pro-inflammatory cytokine (IL-1β, IL-6, and tumor necrosis factor-α [TNF-α]), and lactate dehydrogenase (LDH) concentrations showed significant ($p < 0.05$) fungal species (*S. chartarum* vs. *C. cladosporioides*) and strain (58-17 vs. 58-06), spore dose and time dependent changes. The no adverse effect level (NOAEL) due to exposure to spores of JS 58-17 and JS 58-06 was < 30 spores/g BW; for *C. cladosporioides* it was > 300 spores/g BW. At moderate and high *S. chartarum* doses, BALF composition reflects differences in strain toxicity while at the lowest dose, BALF composition of either *S. chartarum* strain were similar. This suggests that at low spore doses, it is spore sequestered factors common to both strains not strain dependent toxins that are contributing to lung disease onset.**

***Key Words: Stachybotrys chartarum*; spores; inflammation; cytotoxicity; macrocyclic trichothecenes; atranones; intratracheal instillation; mouse.**

It is well known that *Stachybotrys chartarum* (Ehrenb. ex Link) Hughes (= *S. atra* Corda) produces three major classes of secondary metabolites each with varying biological activities (Miller *et al.,* in press). However, it has only been recently recognized that *S. chartarum* can be separated into two chemotypes based on secondary metabolite production profiles (Andersen *et al.*, 2002; Nielsen *et al.*, 2002; Peltola *et al.*, 2002). One chemotype produces macrocyclic trichothecenes; the other produces atranones (and sometimes simple trichothecenes, e.g., trichodermol and trichodermin).

In vitro studies have revealed that the macrocyclic trichothecene producing isolates are highly cytotoxic while the atranone producers are associated with induction of strong inflammatory responses (Nielsen *et al.*, 2002). *In vivo* studies have shown that histopathogical responses of mouse lung exposed to spores of a macrocyclic trichothecene-producing isolate are more severe than those associated with lungs exposed to spores of a nontrichothecene producing isolate (Nikulin *et al.*, 1996, 1997). They have also revealed that exposure to *Stachybotrys* spores (both chemotypes) can result in massive lung damage with acute lethality in both mice (Nikulin *et al.*, 1996, 1997; Rand *et al.*, 2002a) and rats (Yike *et al.*, 2002a). However, the *in vivo* experiments have been generally conducted beyond the maximum tolerated dose, using from about 3000 spores/g body weight (BW) in mice (Nikulin *et al.*, 1996, 1997; Rand *et al.*, 2002a, b) to some 100,000 spores/g BW in rats (Rao *et al.*, 2000, Yike *et al.*, 2002). Not only are these doses higher than those encountered by humans in most contaminated environments (Miller *et al.*, 2003), use of high spore loads makes it unclear whether lung damage is a consequence of the cytotoxic and/or inflammatory toxic properties associated with the spores administered to the animals.

While there is an abundance of information concerning exposure outcome in animals at high spore doses, there is surprisingly little on the effects of low *S. chartarum* spore doses. Rao *et al.* (2000b) revealed that exposure doses of about 3000 spores/g BW represented the no adverse effect level (NOAEL) in Charles River-Dawley rats. However, whether these investigators were using a macrocyclic trichothecene- or an atranone-producing *S. chartarum* isolate is unknown. Interestingly, in mice, exposure to 3000 *S. chartarum* or *Cladosporium cladosporioides* (= nontoxigenic phylloplane fungus) spores/g BW results in a variety of pathological lung changes (Gregory *et al.*, 2002; Rand *et al.*, 2002a, b; Sumarah *et al.*, 1999), indicating that the NOAEL for spores of these two species in mice is lower than it is for *S. chartarum* in Dawley rats. What constitutes the NOAEL for *S. chartarum* exposures

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in mice and whether it is the same for the two *S. chartarum* chemotypes and *C. cladosporioides* is unknown.

The purpose of this study was to use bronchoalveolar lavage fluid (BALF) to investigate dose-response and time-course relationships between exposure to macrocyclic trichothecene- (JS 58-17) and atranone-producing (JS 58-06) *S. chartarum* strains and *Cladosporium cladosporioides* spores, and vascular, inflammatory and/or cytotoxic lung responses in mice. Based on results of our previous studies, we hypothesized that the vascular, inflammatory, and cytotoxic responses in mice exposed to *S. chartarum* spores would be significantly more severe than those associated with *C. cladosporioides* (nontoxigenic) spore exposures. Based on *in vitro* assays (Nielsen *et al.*, 2002), we also hypothesized that cytotoxicity would be significantly more pronounced in mouse lungs exposed to spores of the macrocyclic trichothecene producer compared to that associated with those of the atranone producer. Lastly, based on the *in vitro* studies of Sorenson *et al.* (1987), which showed that the NOAEL for pure satratoxin-H *in vitro* to be about (0.005 μM), and those of Yike *et al.* (1999, 2002a) that showed that spores of the macrocyclic trichothecene producing *S. chartarum* strain we used in our studies to contain 670 fg satratoxin G (SG) equivalents/spore, we hypothesized that the NOAEL for *S. chartarum* exposure in mice would be near the *in vitro* NOAEL for satratoxin H, which is equivalent to the satratoxin H content in about 30 spores/g BW in a 25 g wt. animal.

MATERIALS AND METHODS

The trichothecene producing *Stachybotrys chartarum* isolate employed in this study was from Cleveland (Cleveland strain # JS 58-17 (= ATCC 201211) supplied by Dr. D. Dearborn, Rainbow Babies Children's Hospital, Cleveland, OH). Protein translation inhibition assay revealed this isolate to contain 670fg satratoxin G (SG) equivalents/spore (Yike *et al.*, 1999) while the ELISA revealed it to contain 980fg SG equivalents/spore (Chung *et al.*, in press). The atranone-producing *S. chartarum* (strain # JS 58-06) isolate employed was supplied by Dr. S. Vesper (U.S. Environmental Protection Agency, Cincinnati, OH). The *Cladosporium cladosporioides* isolate was used as a negative control and was recovered from an outdoor sampling site in Nova Scotia (T.G.R.). The two *S. chartarum* strains and *Cladosporium cladosporioides* were cultured on *Stachybotrys* specific medium© (LabCor, Seattle, WA) at 25°C for three weeks prior to instillation.

Spores of all these species were harvested by gently flooding the cultures with sterile physiological saline (0.9% NaCl), followed by gentle agitation to lift the spores but not mycelium from the medium surface. Spores were then collected with a glass pipette and suspended in sterile physiological saline. Spore concentrations were then determined using a hemacytometer and diluted to a working concentration of 1.5×10^6 spores/ml. To minimize toxin loss from spores, spore suspensions were used in the instillation experiments within 1 h of preparation. Previous work has shown that spores of both *S. chartarum* strains suspended in saline for this time will still retain toxins (Gregory *et al.*, 2002; Rand *et al.*, in press).

Animals. Random-bred pathogen-free, Carworth Farms white (CFW), Swiss Webster, male mice, 21–28 days old, (25.3 ± 1.2 g BW) were used in this experiment. The mice were housed according to the standards of the Canadian Council for Animal Care (CCAC, 1993) and with approval from the Saint Mary's University and Dalhousie University Animal Care Committees. The mice were given food and water *ad libitum* and acclimatized for one week prior to use.

Intratracheal instillations. A total of 260 mice were used in this experiment, including ten control (untreated) animals. The treatment animals (*S. chartarum* strains JS 58-17 and JS 58-06, *C. cladosporioides* and saline) were separated into groups of five mice. The mice were then lightly anesthetized with a 0.2 ml im injection of a mixture containing an average of 124.56 mg/kg of ketamine (Ketaleen) and 8.78 mg/kg of xylazine (Rompun) in physiological saline. Once anesthetized, each mouse was weighed to the nearest 0.1 g and placed upright dorsal side down, on an intratracheal instillation board seated 20° from the vertical as described in Mason *et al.* (1998). Each mouse with the exception of untreated control (UTC) mice was instilled with 50 μl of *S. chartarum* (strains 58-17 or 58-06) or *C. cladosporioides* spores or 0.9% NaCl. For the dose dependent study, groups of mice were given spore concentrations at 30, 300, or 3000 spores/g BW. Dilutions were made appropriately from the original stock solution (1.5×10^6 spores/ml). Mice were left in the upright position on the instillation board for approximately 2 min and were then put back into their cages on a warm pad and allowed to recover for 2 h. During recovery, mice were continuously monitored for signs of sickness or distress as outlined in CCAC guidelines (CCAC, 1993).

Bronchoalveolar lavage fluid (BALF) recovery. The treatment mice were killed after 3, 6, 24, 48, and 96 h post instillation (PI) using a 300 μl intraperitoneal injection of 65 mg/ml of sodium pentobarbital (Somnotol). They were immediately weighed to the nearest 0.1g. They were then exsanguinated by cutting the abdominal artery. The mouse lungs were then lavaged with 0.9% physiological NaCl in 4×0.8ml aliquots as previously described in Mason *et al.* (1998). BALF was then distributed into four 1.5 ml microcentrifuge tubes into which an anti-proteinase mixture was added as follows: 100 mM phenylmethylsulfonyl fluoride (PMSF) in iso-propanol was first added to each of the four tubes at a concentration of 10 μl/mlBALF, followed by 500 mM of ethylenediamine tetraacetic acid (EDTA) in ddH_20 per 10 μl/mlBALF. The BALF sample tubes were then flash frozen in liquid N_2 and stored at –36°C.

Total protein, albumin and lactate dehydrogenase (LDH) analysis. Total protein concentration in BALF, an indicator of epithelial and cell membrane integrity, was quantified using the modified Lowry Method Protein Assay Kit (Sigma Chemical Co.). Briefly, this involved a modified trichloro-acetic acid protein precipitation reaction followed by a modified Lowry method for colorimetric concentration determination. Sample absorbance was recorded on a Novaspec Spectrophotometer© at a wavelength of 600 nm. For total protein, BALF samples were diluted 50:50 with dH_20.

Albumin concentrations in BALF, an indicator of vascular permeability, were quantified using a quantitative enzyme linked immunosorbent assay (ELISA) purchased from Bethyl Laboratories, Inc. Samples were diluted 1:800 in diluent (Tris-buffered saline [TBS] with 1% bovine serum albumin [BSA], 0.05% Tween 20, pH 8). Final absorbencies were determined using a Bio-Tek Instruments© ELx800 automated microplate reader at a wavelength of 450 nm.

LDH levels were measured as an indicator of cytotoxicity. Concentrations were determined using the enzyme based Cytotoxicity Detection Kit (Roche Diagnostics). A standard curve was generated for quantification of LDH levels. This was prepared by serially diluting an L-LDH solution (Roche Diagnostics) in physiological 0.9% NaCl to concentrations of 10, 25, 50, 75, 100, 125, and 150 ng/ml. The final absorbance of the samples was determined at the 490 nm wavelength using the microplate reader.

Proinflammatory and oxidative stress cytokine (IL-1β, IL-6, and TNF-α) analysis. Concentrations of the proinflammatory and oxidative stress cytokines IL-6 and TNF-α in BALF were determined using sandwich ELISA procedures purchased from BD Biosciences. Assay diluent and tetramethyl benzoate (TMB) substrate reagent sets were also purchased from BD Biosciences. All standard curves for ELISA kits were generated according to respective kit instructions. IL-1β concentrations were determined with a sandwich ELISA kit purchased from R&D Systems. The assay diluent used was a solution of TBS with 3% BSA and 0.05% Tween 20, pH 7.3). Samples were diluted appropriately in each assay and final absorbances were read at the 450 nm wavelength using the microplate reader.

Statistical analysis. All data were tested for normality using a normal probability plot. Weight measurements followed normal distribution while all other data were transformed to improve normality and homoscedasticity using a log transformation. Significant differences between and amongst treatment groups were determined using one- or two-way ANOVA. The factor for one-way ANOVA was treatment, and for two-way ANOVA the factors were treatment and time. Tukey's multiple comparison test was used to find the location of any differences between means of treatment and control groups. The results are expressed as the mean ± SE. Correlation coefficients for all BALF parameters measured and pooled as a value for each treatment group were determined using Pearson correlation matrix to determine the nature and strength of any relationships. All tests were carried out using Systat version 5.1, and were considered significant at the 0.05 probability level.

RESULTS

Mice exposed to spores of *C. cladosporioides* or *S. chartarum* strains did not show any clinical signs of illness or respiratory distress.

Vascular Leakage

Total protein and albumin concentrations in bronchoalveolar lavage fluid (BALF) from untreated control, saline, and low, moderate, and high spore dose treatment animals are shown in Figure 1. Total protein concentration was significantly increased in mouse lungs exposed to high dose *S. chartarum* strain 58-17 compared to all other treatments ($p \leq 0.001$). Albumin concentrations were significantly increased in mouse lungs exposed to high dose *S. chartarum* strain 58-06 ($p \leq 0.001$) and medium ($p \leq 0.01$) and high ($p \leq 0.001$) dose *S. chartarum* strain 58-17 compared to all other treatments ($p \leq 0.001$). Total protein and albumin concentration profiles were similar and showed dose dependent-like profiles with lowest response levels in low *C. cladosporioides* and *S. chartarum* (both strains) spore dose animals and highest response in high spore dose animals. Figure 1 also indicates that the majority of the increased total protein in the BALF was albumin.

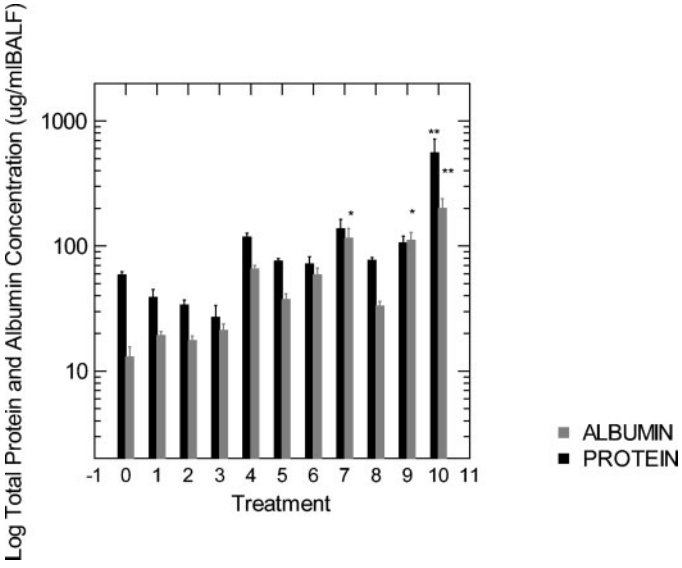

FIG. 1. Pooled data showing dose dependent vascular leakage through changes in both total protein and albumin concentrations in mice BALF. *·**Indicates where concentrations are significantly greater than *C. clad* controls $p \leq 0.01$, $p \leq 0.001$ respectively. (0 = UTC, 1 = saline, 2 = *C. clad*. 30 spores/g BW, 3 = *C. clad*. 300 spores/g BW, 4 = *C. clad* 3000 spores/g BW, 5 = *S. chartarum* 58-06 30 spores/g BW, 6 = *S. chartarum* 58-17 300 spores/g BW, 7 = *S. chartarum* 58-06 3000 spores/g BW, 8 = *S. chartarum* 58-17 30 spores/g BW, 9 = *S. chartarum* 58-17 300 spores/g BW, 10= *S. chartarum* 58-17 3000 spores/g BW).

Figure 2A shows that at the high JS 58-17 spore dose, total protein concentration increased precipitously within 3 h ($p < 0.001$) to highest levels at 24 h PI then declined to near saline and untreated control levels by 96 h PI. Total protein concentration increase in this treatment group was about 10× higher than in all of the other groups treated with highest spore loads for the first 25–30 h. For JS 58-06 and *C. cladosporioides* spore treated animals, total protein concentrations increased gradually from 3 h of exposure, to reach maximal levels at 48 h. They then remained elevated for the duration of the experiment. Total protein concentration at 48 h PI was significantly higher for JS 58-06 than for *C. cladosporioides* ($p < 0.05$) while at 96 h PI it was higher than in all of the other groups ($p < 0.01$). At the moderate dose (300 spores/g BW), protein concentration profiles for JS 58-06 and JS 58-17 were similar to those seen in the highest spore dose exposure, although the mean concentrations were lower (Fig. 2B). Protein concentration profiles in animals exposed to the lowest spore dose (30 spores/g BW) of the *S. chartarum* strains were similar (Fig. 2C) with total protein levels significantly higher throughout the 96 h exposure time ($p < 0.05$) than those in animals instilled with either the *C. cladosporioides* spores or saline.

Proinflammatory Cytokines

Interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) concentrations for all treatment groups were generally highest at 6 h PI and are shown in Table 1. The exceptions were for the IL-1β and TNF-α concentration in mice treated with the two highest doses of *S. chartarum* (both strains), which showed highest levels at 24 h PI. The TNF-α data revealed a dose dependent-like response pattern for the *C. cladosporioides* treated animals, but not for the JS 58-06 or JS 58-17 spore treated animals. In these two last-mentioned treatment groups, highest TNF-α concentrations at 6 h PI were in the groups receiving 300 spores/ g BW. Interleukin-1 (IL-1 β) concentrations at 6 h were significantly increased only in animals exposed to highest JS 58-06 spore load ($p < 0.02$). Interleukin-6 (IL-6) levels at 6 h PI also showed dose dependent like profiles with lowest response levels in low *C. cladosporioides* and *S. chartarum* (both strains) spore dose animals and highest response in high spore dose animals (Table 1). Highest IL-6 production was in animals exposed to the 3000 JS 58-17 spores/ g BW ($p < 0.001$).

TNF-α concentration in BALF from animals exposed to

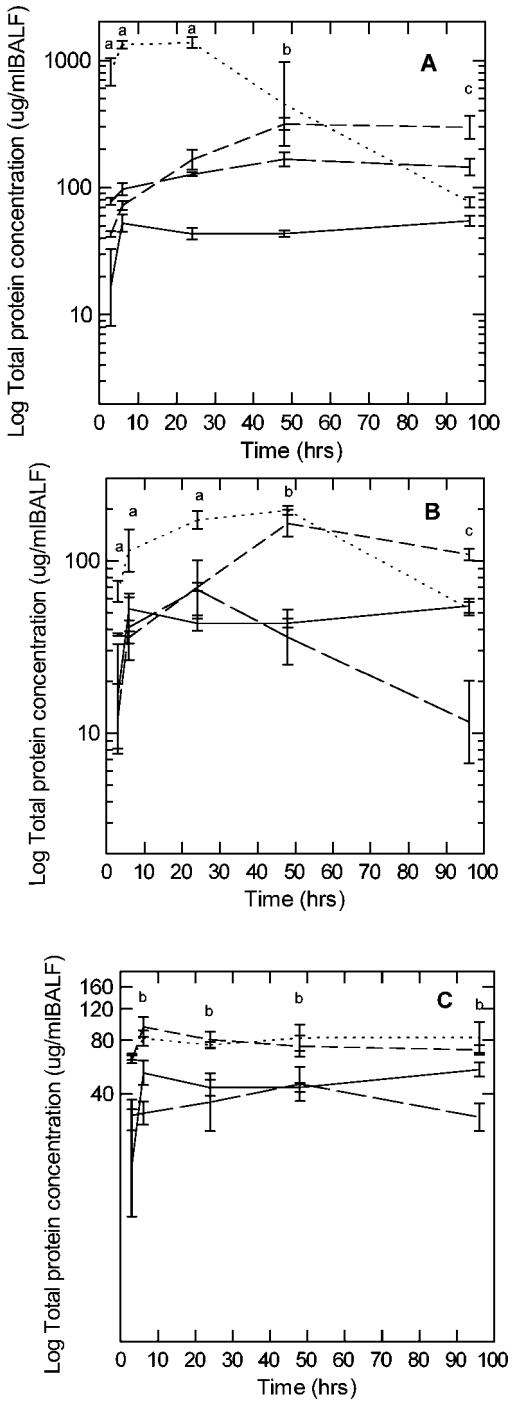

FIG. 2. Time profile for total protein concentration in BALF of mice. A = 3000 spores/g BW, B = 300 spores/g BW, C = 30 spores/g BW. a-*S. chartarum* 58-17 significantly greater than all treatments, b-*S. chartarum* 58-17 and 58-06 significantly greater than all treatments, c-*S. chartarum* 58-06 significantly greater than all treatments. —— saline — — *C. cladosporioides* – – – *S. chartarum* 58-06 - - - - *S. chartarum* 58-17.

highest *C. cladosporioides* and *S. chartarum* spore loads increased rapidly ($p \leq 0.001$) at 3 h compared to that in the saline treated animals (Fig. 3A). Concentration peaked at 6 h in *C. cladosporioides* spore exposed animals, and at 24 h in JS 58-06 and JS 58-17 treated animals. TNF-α levels associated with high dose JS 58-17 spore exposures were lower than those in animals exposed to high *C. cladosporioides* spore loads until 6 h PI ($p \leq 0.05$) and in JS 58-06 exposed animals until 48 h PI ($p < 0.01$). At 96 h PI, TNF-α levels in both *S. chartarum* spore treated animals were similar and significantly higher than in *C. cladosporioides* and saline treated animal groups ($p < 0.05$). In moderate dose JS 58-17 treated animals, TNF-α production peaked ($p < 0.05$) at 24 h followed by decline to nonsignificant levels at 96 h PI. For 58-06 treated animals, TNF-α was highest at 6 h and remained significantly elevated ($p < 0.05$) until 96 h PI (Fig. 3B). TNF-α concentration profiles in animals exposed to the low spore dose of both *S. chartarum* strains were similar. Concentrations were highest at 3 h PI declining to levels similar to *C. cladosporioides* and saline treated animals ($p > 0.05$) by 48 h for JS 58-17, and by 96 h PI for JS 58-06 (Fig. 3C).

IL-1β concentrations in animals exposed to the highest doses of *S. chartarum* (both strains) increased rapidly to highest levels at 24 h PI ($p < 0.01$; Fig. 4A). Thereafter, concentrations in animals exposed to JS 58-06 spores remained significantly elevated ($p < 0.05$) compared to all other treatments for 96 h PI, while those in JS 58-17 exposed animals declined to nonsignificant levels by 48 h PI. IL-1β concentration profiles in animals exposed to the moderate and low doses of *S. chartarum* (both strains) were similar (Figs. 4B and 4C). In these groups, IL-1β concentrations increased rapidly and peaked within 6 to 24 h PI for JS 58-17, or 24 to 48 h for JS 58-06 ($p < 0.05$), followed by decline to nonsignificant levels within 96 h PI.

IL-6 concentration profiles in animals receiving high, moderate, and low doses of fungal spores (Figs. 5A–5C) were similar. At all spore doses, IL-6 concentrations increased rapidly to highest levels within 6 h PI then declined and leveled off at about 24 h PI. However, IL-6 concentration was highest ($p < 0.001$) in animals exposed to the highest JS 58-17 spore dose, and about 10× higher than in the other treatment groups. It was also significantly elevated ($p < 0.05$) but only in animals instilled with low JS 58-17 spore dose at 6 h PI (Fig. 5C).

Cytotoxicity

At highest spore loads, LDH concentration in JS 58-17 treated animals reached maximal levels ($p < 0.05$) at 24 h PI then declined to nonsignificant levels starting at 48 PI. In the JS 58-06 spore exposed animals LDH gradually increased from 3 h PI to significant levels at 48 h PI ($p < 0.05$) and maximal levels at 96 h ($p < 0.01$; Fig. 6A). LDH concentration pattern in *C. cladosporioides* exposed animals was similar to that of JS 58-17, although significant LDH changes were not detected. LDH concentrations in low dose *S. chartarum* (both strains) exposures were similar. Compared to the saline and *C. cladosporioides* spore treated animals, LDH in the *S. chartarum*

TABLE 1
Mean Cytokine Concentrations in BALF for All Treatments at 6 h PI

Treatment	Cytokine (pg/ml BALF)		
	TNF-α	IL-1β	IL-6
UTC	6 ± 3 (n = 10)	8 ± 1 (n = 10)	11 ± 3 (n = 10)
Saline	49 ± 20 (n = 5)	51 ± 17 (n = 4)	45 ± 14 (n = 5)
C. cladosporioides (spores/g BW)			
30	15 ± 3 (n = 5)	32 ± 17 (n = 6)	19 ± 4 (n = 6)
300	388 ± 161 (n = 5)*	46 ± 15 (n = 5)	48 ± 9 (n = 5)
3000	1175 ± 221 (n = 5)**	58 ± 43 (n = 5)	291 ± 46 (n = 5)*
S. chartarum JS 58–06 (spores/g BW)			
30	1020 ± 451 (n = 5)**	173 ± 58 (n = 5)	57 ± 33 (n = 5)
300	1191 ± 262 (n = 4)**	97 ± 17 (n = 5)	105 ± 30 (n = 5)
3000	854 ± 134 (n = 5)**	157 ± 22 (n = 5)*	189 ± 38 (n = 5)*
S. chartarum JS 58–17 (spores/g BW)			
30	270 ± 138 (n = 5)*	200 ± 90 (n = 5)	111 ± 20 (n = 5)
300	358 ± 194 (n = 5)*	46 ± 30 (n = 5)	80 ± 25 (n = 5)
3000	173 ± 60 (n = 5)	63 ± 25 (n = 5)	1733 ± 320 (n = 4)**

Note. Data are expressed as mean ± SE.
*,**Indicates where concentrations are significantly greater than saline treated animals ($p < 0.05$ and $p < 0.001$, respectively).

treated animals increased to significantly elevated levels ($p < 0.01$) at 6 h PI and maximal levels ($p < 0.001$) at 96 h PI.

Pearson correlation coefficient results for the pooled BALF data from high, moderate, and low spore dose treatment groups are summarized in Table 2. They reveal that the strongest positive associations were between protein and albumin ($r = 0.757$), protein and IL-6 ($r = 0.682$), and IL-6 and albumin ($r = 0.510$). The weakest association was between LDH and IL-6 ($r = 0.012$).

DISCUSSION

This study has revealed that there are marked differences in vascular, inflammatory, and cytotoxic lung responses in mice intratracheally exposed to *Cladosporium cladosporioides* or *Stachybotrys chartarum* spores even at low spore doses. This result was not unexpected since previous studies have revealed significant differences in lung cyto- and histopathology (Rand *et al.*, 2002, in press), associated with exposures to spores of these two species. Nevertheless, it was interesting that the NOAEL for the two species was so different. While the NOAEL for *C. cladosporioides* was between 300 and 3000 spores/g BW, it was less than 30 spores/g BW for both *S. chartarum* strains. This result, combined with those of the previous studies showing that the magnitude as well as temporal patterns of biochemical, anatomical, and gross pathological lung responses toward exposures to both of these spore types in mice is different (Gregory *et al.*, 2002; Mason *et al.*, 1998, 2001; McCrae *et al.*, 2001; Rand *et al.*, 2002, in press), further supports the position that exposure outcome is largely attributable to differences in spore toxicity. However, results showing that exposure of mice to the high *C. cladosporioides* but not to high *S. chartarum* 58-17 spore dose stimulated rapid TNF-α production also indicates that factors other than toxins may be involved. Jussila *et al.* (2002b) reported high TNF-α production in the BALF of mice exposed to *Penicillium spinulosum*, a nontoxigenic species, which they attributed to the high β1, 3 glucan content in the spore walls of this species. Whether differences in β1, 3 glucan content in the walls of spores of the two species accounts for at least some of the lung response differences is unknown and deserves further consideration.

The fact that we found the NOAEL response for *S. chartarum* exposures (both chemotypes) in mice to be less than 30 spores/g BW was surprising since Rao *et al.* (2000b) reported the NOAEL to be about 3000 *S. chartarum* spores/g BW in rats. This difference in result may reflect differences in animal species susceptibility (Ueno, 1983). Additionally, it may also reflect differences in degree of sensitivity of the assays used in the respective studies. Rao *et al*. (2000b) did not use ELISA assays but instead more insensitive biochemical and microscopic assays for inflammation such as hemoglobin and leukocyte differential counts, respectively.

Lung response patterns toward exposures to high and moderate spore doses of the two *S. chartarum* chemotypes also differed widely. While vascular, inflammatory, and cytotoxic responses in *C. cladosporioides* spore treated animals showed dose dependent-like responses in all parameters tested except IL-1, for both *S. chartarum* strains, responses showed dose dependency for only total protein, albumin, and IL-6. Dose dependent-like response patterns are not unusual and appear to reflect the magnitude of stimulation, especially in single exposure studies. They have been demonstrated in a variety of *in vivo* animal exposures to nonbiological particulates, bacteria,

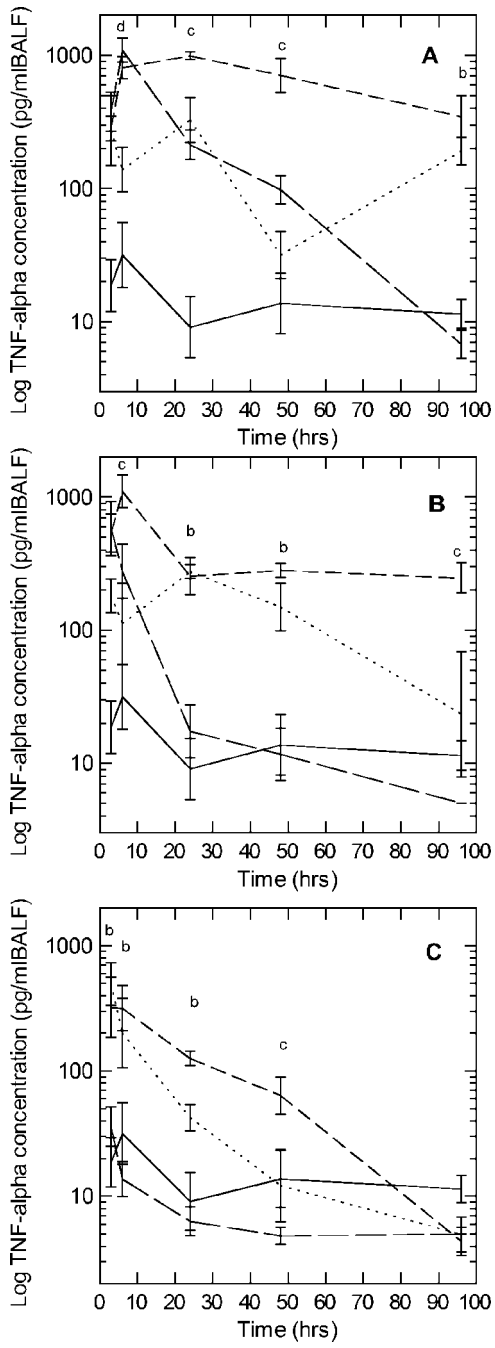

FIG. 3. Time profile for TNF-α concentration in BALF of mice. A= 3000 spores/g BW, B = 300 spores/g BW, C = 30 spores/g BW. a-*S. chartarum* 58-17 significantly greater than all treatments, b-*S. chartarum* 58-17 and 58-06 significantly greater than all treatments, c-*S. chartarum* 58-06 significantly greater than all treatments, d-*S. chartarum* 58-17 and 58-06 and *C. cladosporioides* significantly greater than saline. —— saline — — *C. cladosporioides* – – – *S. chartarum* 58-06 - - - - *S. chartarum* 58-17.

and fungi (Finch *et al.*, 1998; Jussila *et al.*, 2001, 2002a,b,c; Ruotsalainen *et al.*, 1998). Increased total protein and albumin concentrations in BALF are indicative of vascular leakage (Rhoades and Pflanzer, 1996) and have been reported in mice (Mason *et al.*, 2001) and rats (Rao *et al.*, 2000a, b) exposed to *S. chartarum* spores. Rand *et al.* (2002b) proposed that increased total protein concentration in BALF might be due to

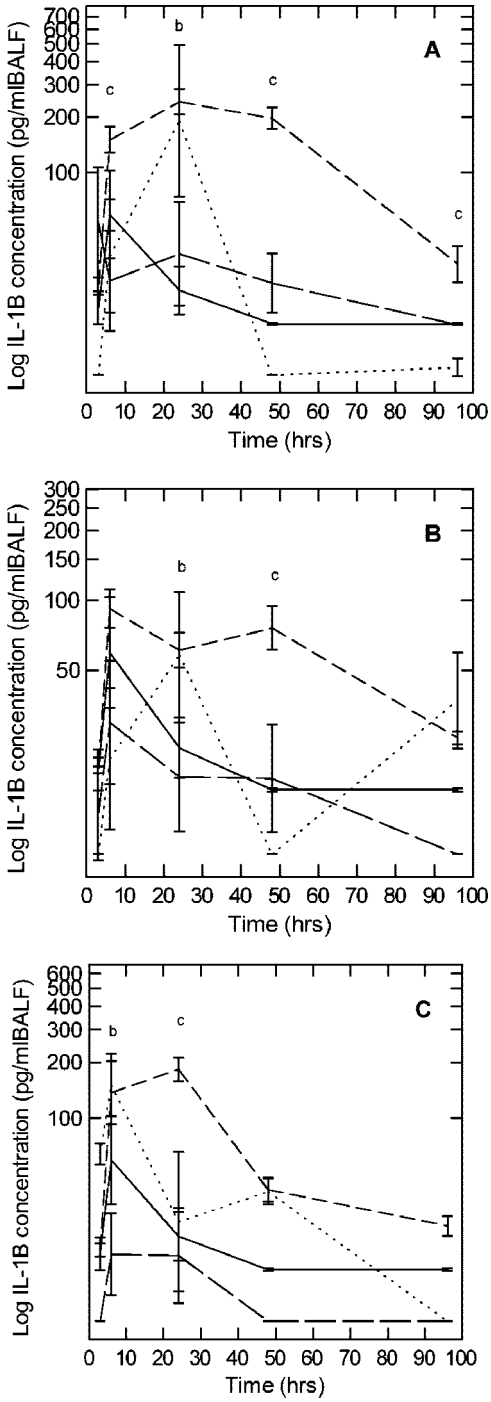

FIG. 4. Time profile for Il-1β concentration in BALF of mice. A = 3000 spores/g BW, B = 300 spores/g BW, C = 30 spores/g BW. a-*S. chartarum* 58-17 significantly greater than all treatments, b-*S. chartarum* 58-17 and 58-06 significantly greater than all treatments, c-*S. chartarum* 58-06 significantly greater than all treatments. —— saline — — *C. cladosporioides* – – – *S. chartarum* 58-06 - - - - *S. chartarum* 58-17.

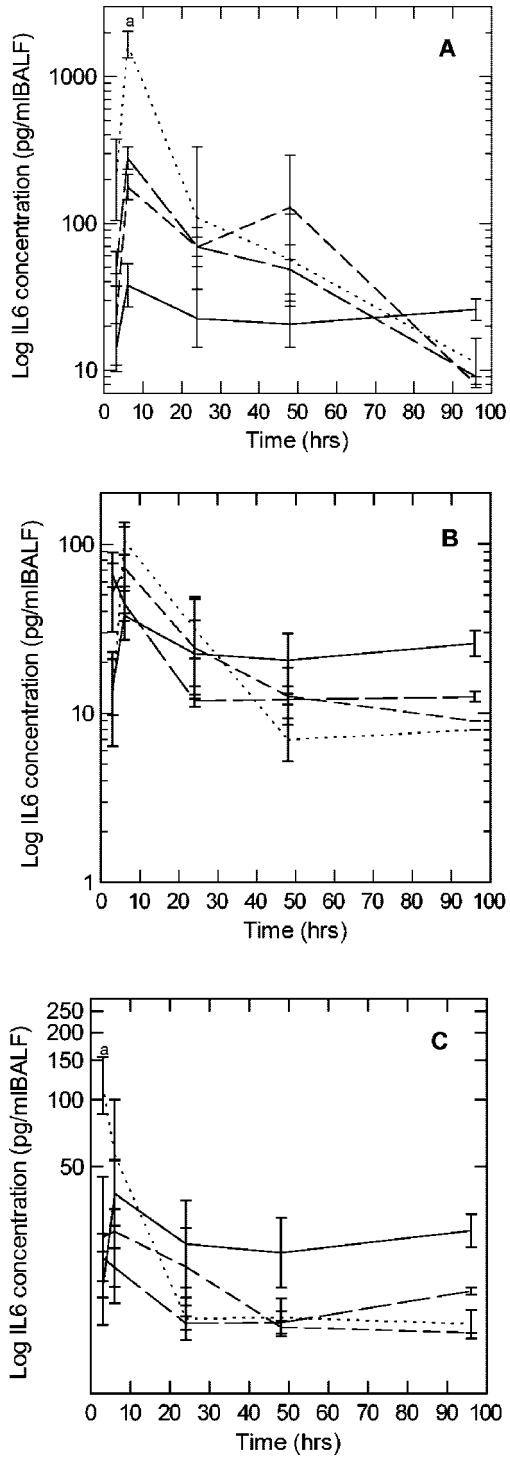

FIG. 5. Time profile for Il-6 concentration in BALF of mice. A = 3000 spores/g BW, B = 300 spores/g BW, C = 30 spores/g BW. a-*S. chartarum* 58-17 significantly greater than all treatments. —— saline — — *C. cladosporioides* – – – *S. chartarum* 58-06 - - - - *S. chartarum* 58-17

granuloma formation resulting in increased blood perfusion pressure and vascular deficit in the affected lung. However, that significant protein exudation into BALF was within 3 h PI

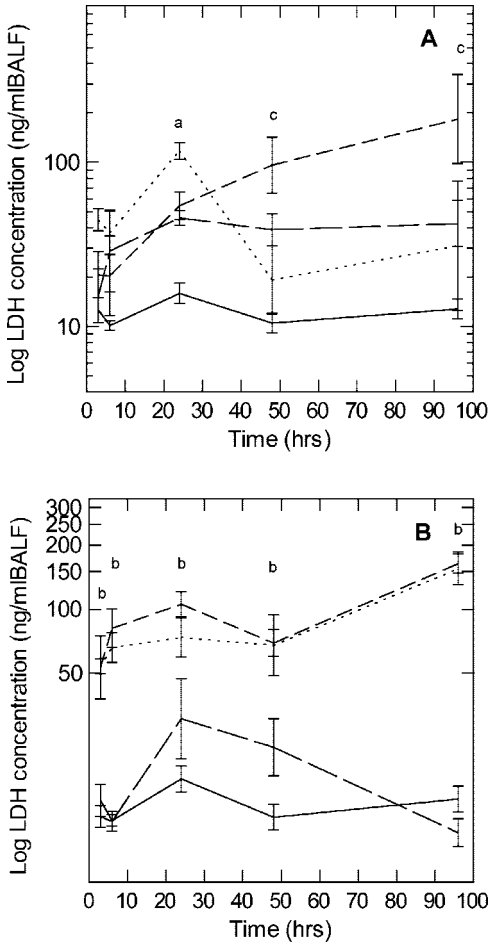

FIG. 6. Time profile for LDH concentration in BALF of mice. A= 3000 spores/g BW, B = 30 spores/g BW. a-*S. chartarum* 58-17 significantly greater than all treatments, b- *S. chartarum* 58-17 and 58-06 significantly greater than all treatments, c- *S. chartarum* 58-06 significantly greater than all treatments, d- *S. chartarum* 58-17 and 58-06 and *C. cladosporioides* significantly greater than saline. —— saline — — *C. cladosporioides* – – – *S. chartarum* 58-06 - - - - *S. chartarum* 58-17.

whereas granuloma formation is apparent only after 12–24 h PI (Rand *et al.*, 2002b) suggests that protein exudation may be due to some endogenously derived substances produced in the lung. The strong correlation between total protein and albumin

TABLE 2
Pearson Correlation Matrix for All Inflammatory Parameters

Factor	Protein	Albumin	IL-1β	IL-6	TNF-α	LDH
Protein	1.00	—	—	—	—	—
Albumin	0.757	1.00	—	—	—	—
IL-1β	0.377	0.340	1.00	—	—	—
IL-6	0.682	0.510	0.226	1.00	—	—
TNF-α	0.089	0.122	0.427	0.146	1.00	—
LDH	0.175	0.418	0.187	0.012	0.085	1.00

production and IL-6 concentration in BALF suggests that this cytokine may be involved. IL-6 is produced by activated alveolar macrophages, fibroblasts, and endothelial cells and results in increased vascular permeability when these cells are stimulated by foreign particulates (Kuby, 1997). Whether other factors, such as endothelins, are associated with increased vascular permeability in lungs exposed to *S. chartarum* spores is unknown and deserves further attention.

While dose dependent-like response patterns were evident for total protein, albumin, and IL-6 levels, they were lacking for LDH in *S. chartarum* treated animals (both strains). LDH is a biomarker of cell damage and death. Detection of highest LDH concentrations in BALF from animals receiving lowest spore loads indicates that even low spore doses are potently cytotoxic. Moreover, that LDH production was a response in animals exposed to spores of both *S. chartarum* chemotypes does not support the position that the macrocyclic trichothecene producing strain (58-17) is more cytotoxic than the atranone producer (58-06).

Dose dependent-like responses were also lacking for IL-1β and TNF-α concentrations in the JS 58-17 spore treated animals and for IL-1β in the JS 58-06 instilled animals. Differences between dose dependency profiles for the pooled TNF-α production in mice exposed to the two *S. chartarum* strains provides evidence that disease outcome towards both strains is different. These response differences may reflect differences in the potency and pharmacokinetics of the toxins found in the spores of each of these strains. For example, the macrocyclic trichothecene toxins sequestered in spores of JS 58-17 are amongst the most potent protein synthesis inhibitors known (Riley and Norred, 1996). These toxins are also lost quickly (within minutes) from spores (Hinkley and Jarvis, 2000) and incorporated into lung cells including alveolar macrophages whereupon they bind to ribosomes (Rand *et al.*, in press). *In vitro* studies have clearly demonstrated that these toxins suppress cytokine synthesis in macrophages (Nielsen *et al.*, 2002; Sorenson *et al.*, 1987). Given that the activated macrophage is the predominant source of both IL-1β and TNF-α *in vivo*, (Kuby, 1997) exposure to high enough concentrations of these toxins may result in decreased cytokine expression. Differences in disease outcome, especially inflammatory responses, toward both *S. chartarum* strains were also seen in the temporal concentration changes of cytokines. What is clear from these data is that at moderate and high spore doses, *S. chartarum* JS 58-17 spores evoked relatively fast lung responses, generally within 24 h PI followed by decline. *Stachybotrys chartarum* JS 58-06 spore exposure stimulated responses, the magnitude of which either increased throughout the 96 h period (total protein, albumin, LDH) or remained at significantly elevated levels for this time period (IL-1β, TNF-α). Differences in temporal responses may have to do with the nature of the pharmacokinetics of each of the toxins. While trichothecenes are considered to act on cells rapidly leading to cell death or to depressed protein synthesis, at least one of the toxins sequestered in spores of JS 58-06, stachylysin, is known to act slowly causing cell membrane disruption after more long-lasting exposure (Vesper *et al.*, 2001).

An important result of the study was finding that exposure to low spore doses (30 spores/g BW) of the two *S. chartarum* strains still precipitated responses that were significantly higher than those associated with *C. cladosporioides* and saline exposures. However, differences in the inflammation response in mice towards the two *S. chartarum* strains at the low spore concentration are unapparent. This result is interesting because concentrations of macrocyclic trichothecenes in the 30 spore/g BW exposure of *S. chartarum* JS 58-17 are less than that associated with the NOAEL in *in vitro* exposures (Sorenson *et al.*, 1987). The commonalities in the response profiles towards the lowest dose suggest that chemicals other than trichothecenes, atranones, and hemolysins sequestered in spores may be contributors of lung pathogenesis. Possibly, these substances include proteinases. Kordula *et al.* (2002) recently reported that *S. chartarum* spores contain high concentrations of chymotrypsin-like serine proteinases, which they identified as stachyrase A. Yike *et al.* (2002b) reported that the spores of both *S. chartarum* JS 58-17 and JS 58-06 contain high concentrations of proteinases although they did not fully characterize them. These proteinases reported by Kordula *et al.* (2002) and Yike *et al.* (2002b) have been shown to cleave a number of collagen types and other structural proteins found in the lung environment (Kordula *et al.*, 2002). Rand *et al.* (2002b) also reported depressed collagen iv expression in lung granulomas surrounding *S. chartarum* spores, which they attributed to serine proteinase activity. Serine proteinases are associated with some entomopathogenic fungal diseases (Khachatourians, 1996), as well as invasive pulmonary mycosis caused by *Aspergillus fumigatus* (Washburn, 1996). These enzymes may also be contributing to some of the inflammatory responses in the animals exposed to low *S. chartarum* spore doses. This obviously deserves attention owing to its implication in understanding lung disease onset associated with *S. chartarum* spore exposures in humans and animals.

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REFERENCES

Andersen, B., Nielsen, K. F., and Jarvis, B. B. (2002). Characterization of *Stachybotrys* from water-damaged buildings based on morphology, growth and metabolite production. *Mycologia* **94,** 392–403.

CCAC (1993). *Guide to the Care and Use of Experimental Animals,* Vol. 1. Bradda Printing Services, Ottawa.

Chung, Y.-J., Jarvis, B. B., Tak, H., and Pestka, J. J. (in press). Immunochemical assay for satratoxin G and other macrocyclic trichothecenes associated with *Stachybotrys chartarum*. *Toxicol. Mech. Method.*

Finch, G. L, Nikula, K. J., and Hoover, M. D. (1998). Dose-response relationships between inhaled berylium metal and lung toxicity in C3H mice. *Toxicol. Sci.* **42,** 36–48.

Gregory, L., Rand, T. G., Dearborn, D., Yike, I., and Vesper, S. (2002). Immunocytochemical localization of a hemolysin-like protein in *Stachybotrys chartarum* spores and spore-impacted mouse and rat lung tissues. *Mycopathologia* **56,** 77–85.

Hinkley, S. F., and Jarvis, B. B. (2000). Method for *Stachybotrys* toxins. In *Methods in Molecular Biology: Mycotoxin Protocols* (M. W. Trucksess, and A. E. Pohland, Eds.), Vol. 157, pp. 173–194. Humana Press.

Jussila, J., Komulainen, H., Huttunen, K., Roponen, M., Halinen, A., Hyvarinen, A., Kosma, V. M., Pelkonen, J., and Hirvonen, M. R. (2001). Inflammatory responses in mice after intratracheal instillation of spores of *Streptomyces californicus* isolated from indoor air of a moldy building. *Toxicol. Appl. Pharm.* **171**(1), 61–69.

Jussila, J., Komulainen, H., Huttunen, K., Roponen, M., Iivanainen, E., Torkko, P., Kosma, V. M., Pelkonen, J., and Hirvonen, M. R. (2002a). *Mycobacterium terrae* isolated from indoor air of a moisture-damaged building induces sustained biphasic inflammatory response in mouse lungs. *Environ. Health Persp.* **110(11),** 1119–1125.

Jussila, J., Komulainena, H., Kosma, V., Pelkonen, J., and Hirvonen, M. (2002b). Inflammatory potential of the spores of *Penicillium spinulosum* isolated from indoor air of a moisture-damaged building in mouse lungs. *Environ. Toxicol. Phar.* **12**(3), 137–145.

Jussila, J., Kosma, V. M., Komulainen, H., Nevalainen, A., Pelkonen, J., and Hirvonen, M. (2002c). Spores of *Aspergillus versicolor* isolated from indoor air of a moisture-damaged building provoke acute inflammation in mouse lungs. *Inhal. Toxicol.* **14**(12), 1261–1277.

Khachatourians, G. G. (1996). Entomopathogenic fungi. In *The Mycota: Human and Animal Relationships* (D. H. Howard and J. D. Miller, Eds.), pp. 331–357. Springer-Verlag, Berlin Hiedelberg. Germany.

Kordula, T., Banbula, A., Macomson, J., and Travis, J. (2002). Isolation and properties of stachyrase A, a chymotrypsin-like serine proteinase from *Stachybotrys chartarum*. *Infect. Immun.* **70**(1), 419–421.

Kuby, J. (1997). Leukocytes migration and inflammation. In *Immunology*, 3rd ed. (Eds.), pp. 357–378. W. H. Freeman.

Mason, C. D., Rand, T. G., Oulton, M., MacDonald, J. M., and Scott, J. E. (1998). Effects of *Stachybotrys chartarum* (*atra*) conidia and isolated toxin on lung surfactant production and homeostasis. *Nat. Toxins* **6,** 27–33.

Mason, C., Rand, T. G., Oulton, M., and MacDonald, J. (2001). The effect of *Stachybotrys chartarum* spores and an isolated trichothecene, isosatratoxin F, on convertase activity in mice. *Toxicol. Appl. Pharm.* **72,** 21–28.

McCrae, K. C., Rand, T., Shaw, R. A., Mason, C., Oulton, M. R., Hastings, C., Cherlet, T., Thliveris, J. A., Mantsch, H. H., MacDonald, J., and Scott, J. E. (2001). Analysis of pulmonary surfactant by Fourier-transform infrared spectroscopy following exposure to *Stachybotrys chartarum* (*atra*) spores. *Chem. Phys. Lipids.* **110,** 1–10.

Miller, J. D., Rand, T. G., and Jarvis, B. (in press). *Stachybotrys chartarum*: Cause of human disease or media darling. *Med. Mycol.*

Nielsen, K. F., Huttunen, K., Hyvarinen, A., Andersen, B., Jarvis, B. B., and Hirvonen, M. R. (2002). Metabolite profiles of *Stachybotrys* isolates from water-damaged buildings and their induction of inflammatory mediators and cytotoxicity in macrophages. *Mycopathologia* **154,** 201–205.

Nikulin, M., Reijula, K., Jarvis, B. B., and Hintikka, E.-L. (1996). Experimental lung mycotoxicosis in mice induced by *Stachybotrys atra*. *Int. J. Exp. Pathol.* **77,** 213–218.

Nikulin, M., Reijula, K., Jarvis, B., Veijalainen, P., and Hintikka, E.-L. (1997). Effects of intranasal exposure to *Stachybotrys atra*. *Fund. Appl. Toxicol.* **35,** 182–188.

Peltola, J., Niessen, L., Nielsen, F., Jarvis, B. B., Andersen, B., Salkinoja-Salonen, M., and Moller, E. M. (2002). Toxigenic diversity of two different RAPD groups of *Stachybotrys chartarum* isolates analyzed by potential for trichothecene production and for boar sperm cell motility inhibition. *Can. J. Microbiol.* **48,** 1017–1029.

Rand, T. G., Gregory, L., Pestka, P., and Dearborn, D. (2002a). Satratoxin in *Stachybotrys chartarum* spores and spore impacted mouse lungs. *Toxicol. Pathol*.

Rand, T. G., Mahoney, M., White, K., and Oulton, M. (2002b). Microanatomical changes associated with alveolar type II cells in juvenile mice exposed to *Stachybotrys chartarum* and isolated toxin. *Toxicol. Sci.* **65,** 239–245.

Rand, T. G., White, K., Logan, A., and Gregory, L. (2002c). Histological, immunohistochemical and morphometric changes in lung tissue in juvenile mice experimentally exposed to *Stachybotrys chartarum* spores. *Mycopathologia* **56,** 87–99.

Riley, R. T., and Norred, W. P. (1996). Mechanisms of mycotoxicity. In *The Mycota* (H. Howard and J. D. Miller, Eds.), vol. VI, pp. 193–211. Springer-Verlag, New York.

Rao, C. Y., Brain, J. D., and Burge, H. A. (2000a). Reduction of pulmonary toxicity of *Stachybotrys chartarum* spores by methanol extraction of mycotoxins. *Appl. Environ. Microbiol.* **66,** 2817–2821.

Rao, C. Y., Burge, H. A., and Brain, J. A. (2000b). The time course of responses to intratracheally instilled toxic *Stachybotrys chartarum* spores in rats. *Mycopathologia* **149,** 27–34.

Rhoades, R., and Pflanzer, R. (1996). *Human Physiology*. Saunders, Toronto.

Ruotsalainen, M., Hirvonen, M. R., Nevalainen, A., Meklin, T., and Savolainen, K. (1998). Cytotoxicity, production of reactive oxygen species and cytokines induced by different strains of *Stachybotrys* sp. from mouldy buildings in RAW2647 macrophages. *Environ. Toxicol. Phar.* **6,** 193–199.

Sorenson, W. G., Frazer, D. G., Jarvis, B., Simpson, J., and Robinson, V. A. (1987). Trichothecene mycotoxins in aerosolized conidia of *Stachybotrys atra*. *Appl. Environ. Microbiol.* **53,** 1370–1375

Sumarah, M. W., Rand, T. G., Mason, C. D., Oulton, M., MacDonald, J., and Anthes, M. (1999). Effects of *Stachybotrys chartarum* spores and toxin on mouse lung surfactant phospholipid composition. Proceedings of the Third International Conference on Bioaerosols, fungi and mycotoxins. Health Effects, Assessment, Prevention and control. Saratoga Springs, NY. pp. 444–452.

Ueno, Y. (1983). *Trichothecenes: Chemical, Biological and Toxicological Aspects.* Elsevier, New York.

Vesper, S. J., Magnuson, M. L., Dearborn, D. G., Yike, I., and Haugland, R. A. (2001). Initial characterization of the hemolysin stachylysin from *Stachybotrys chartarum*. *Infect. Immun.* **69,** 912–916.

Washburn, R. G. (1996). Opportunistic mold infections. In *The Mycota: Human and Animal Relationships* (D. H. Howard and J. D. Miller, Eds.), pp. 147–158. Springer-Verlag, Berlin Hiedelberg, Germany.

Yike, I., Allan, T., Sorenson, W. G., and Dearborn, D. G. (1999). Highly sensitive protein translation assay for trichothecene toxicity in airborne particulates. Comparison with cytotoxicity assays. *Appl. Environ. Microbiol.* **65,** 88–94.

Yike, I., Miller, M. J., Tomashefski, J., Walenga, R., and Dearborn, D. (2002a). Infant rat model of *Stachybotrys chartarum* induced mycotoxicosis. *Mycopathologia* **154,** 139–152

Yike, I., Rand, T.,, and Dearborn, D. (2002b). Proteases from the spores of toxigenic fungus *Stachybotrys chartarum. Amer. J. Respir. Crit. Care Med*. **165**(Suppl. American Thoracic Soc. Abstracts), A537