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Effects of Intranasal Exposure to Spores of *Stachybotrys atra* in Mice

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The effects of highly toxic and nontoxic spores of *Stachybotrys atra* were investigated in mice after six intranasal administrations of 1×10^5 and 1×10^3 spores in phosphate-buffered saline during a 3-week period. Toxic spores contained the trichothecene mycotoxins, satratoxins G and H, as well as the immunosuppressant stachybotrylactones and -lactams. No trichothecenes were detected in the nontoxic spores, and they contained only minor amounts of stachybotrylactones and -lactams. In mice injected with toxic and nontoxic spores, the platelet count was decreased and leucocyte and erythrocyte counts, hemoglobin concentration, and hematocrit were increased. No IgG antibodies to *S. atra* were detected in sera of mice exposed intranasally to spores. No histological changes were detected in spleen, thymus, or intestines of mice. The mice receiving 1×10^5 toxic spores intranasally developed severe inflammatory changes within both bronchioles and alveoli. Hemorrhage was detected in alveoli. The mice receiving 1×10^5 nontoxic spores also developed inflammatory changes in the lungs, but these changes were significantly milder than those in mice receiving toxic spores. The mice receiving 1×10^3 toxic spores developed inflammatory changes in the lungs that were less severe than those in the mice receiving 1×10^5 toxic spores. No inflammatory changes were detected in the mice receiving 1×10^3 of nontoxic spores. The present findings indicate that exposure to *S. atra* spores containing toxins (satratoxins) can be a significant health risk.

The fungus *Stachybotrys atra* (synonym of *Stachybotrys chartarum*) has been isolated from building materials and air samples in buildings with moisture problems (Bisset, 1987; Croft *et al.*, 1986; Hunter *et al.*, 1988; Johanning *et al.*, 1996; Miller *et al.*, 1988). Many strains of *S. atra* can produce highly toxic secondary metabolites, trichothecenes, and other compounds (Jarvis, 1991; Jarvis *et al.*, 1995), while some of the strains are only slightly toxic (Korpinen and Uoti, 1974). The fungus can grow and produce nonvolatile mycotoxins on cellulose-containing building materials, such as wallpaper and gypsum board liner, at high moisture content (Nikulin *et al.*, 1994). In buildings contaminated with *S. atra*, residents have suffered from cough; irritation of the eyes, skin, and mucous membranes; respiratory symptoms, headache, and fatigue (Croft *et al.*, 1986; Johanning *et al.*, 1996). Spores of *Stachybotrys* can contain toxins (Sorenson *et al.*, 1987). Most of the *S. atra* isolates are able to produce the potent cytotoxic satratoxins (Bata *et al.*, 1985; Harrach *et al.*, 1983; Jarvis *et al.*, 1986), which belong to a group of macrocylic 12,13-epoxytrichothec-9-enes (Jarvis, 1991).

One of the most widely studied trichothecene mycotoxins is T-2 toxin, a toxin produced by some species of *Fusarium*. T-2 toxin is one of the most toxic trichothecenes studied and can alter the immune system in animals (WHO, 1990). Satratoxin H, a mycotoxin closely related in structure to T-2 toxin and containing a macrocylic ring, is able to cause lesions in the lymphoid organs. Lymphocyte depletion in the thymus and lymph nodes caused by satratoxin H is similar to lesions caused by T-2 toxin in mice by intraperitoneal administration. Satratoxin H, however, has been up to five times more toxic than T-2 toxin in animal experiments (Glávits and Ványi, 1988).

In experimental studies mice, rats, guinea pigs, and pigs have been exposed to aerosolized particles of purified T-2 mycotoxin (Creasia *et al.*, 1987; 1990; Marrs *et al.*, 1986; Pang *et al.*, 1987). Inhalation exposure to the toxin caused necrosis of spleen, thymus, and lymphoid tissue as well as epithelial necrosis in the intestines. These changes were similar to those following experimental administration by the parenteral route. Only minor or no changes could be detected in lung tissue (Creasia *et al.*, 1987; Marrs *et al.*, 1986; Pang *et al.*, 1987). Inhalation of T-2 toxin was at least 10 times more toxic than systemic administration and at least 20 times more toxic than dermal administration of the toxin to mice (Creasia *et al.*, 1987). Toxin-containing spores act as carriers of toxins in environments contaminated with *S. atra* resulting in exposure to airborne mycotoxins. So far, inhalation studies have not been carried out with spores containing mycotoxins to evaluate changes in the respiratory tract.

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The aim of the present study was to investigate the histological changes in lungs, spleen, thymus, and intestines of mice after exposure to spores of highly toxic and nontoxic strains of *S. atra,* a fungus frequently isolated from houses with moisture problems. Spore suspensions were administered intranasally twice a week to mice during the 3-week experiment. Systemic effects of the intranasal administration of *S. atra* spores were evaluated by investigating changes in hematological parameters and measuring IgG antibodies to *S. atra* antigen. Intranasal administration in mice has been used previously in developing animal models of allergic bronchopulmonary aspergillosis and latex allergy (Kurup *et al.,* 1992; 1994). In previous experiments, intranasal administration has proved to be a reliable method for introducing antigens into the lungs of anesthetized animals.

MATERIALS AND METHODS

Stachybotrys atra strains and toxins in spore material. Two *S. atra* strains (s. 72 and s. 29) were cultured on rice flour agar (Samson and van Reenen-Hoekstra, 1988), first for 2 weeks at 20–23°C and then for 2 weeks at 6–8°C. Spores were suspended from agar to phosphate-buffered saline (PBS, pH 7.2) Biological toxicity in a feline fetus lung cell line was determined, and the chemical analysis of *Stachybotrys* toxins was performed as described previously (Nikulin *et al.,* in press). In feline fetus lung cells, strain s. 29 proved to be only slightly toxic (0.8 mg crude extract/ml caused cell death), whereas strain s. 72 was extremely toxic (60 ng crude extract/ml caused cell death); strain s. 72 was about 13,000-fold more toxic than strain s. 29. The spores of strain s. 72 contained satratoxins G and H, stachybotrylactone, and stachybotrylactam at 4 ng, 10 ng, 8 μg, and 2 μg/10^5 spores, respectively. No satratoxins were detected in spores of the strain s. 29 and only minor amounts of stachybotrylactone and stachybotrylactam (<1 ng/10^5 spores) were detected. Strains are subsequently referred to as toxic (s. 72) and nontoxic (s. 29).

Intranasal administration. Both female and male pathogen-free NMRI mice (National Veterinary and Food Research Institute, Helsinki, Finland), age 5 weeks, average body weight 21.8 ± 1.7 g, were used in the experiment. The mice were divided into five groups (10 animals in each group: 5 females and 5 males). Mice were housed five per polycarbonate cage, with free access to food and water. Mice were held in a room maintained at 20–23°C, 50% relative humidity, 12 room air changes per hour, and a 12-hr photoperiod. Two groups received intranasally (i.n.) 50 μl 1×10^5 or 1×10^3 spores of nontoxic strain (s. 29) in PBS, while the other two groups received 1×10^5 or 1×10^3 spores of toxic strain (s. 72). One group served as a control, receiving 50 μl PBS. The mice were anesthetized with ether before the i.n. instillation of spores or PBS. Small drops of spore solution were instilled into the nostrils of mice using a pipet tip. The aspiration of solution into the respiratory tract was visually evaluated. The mice were held in an upright position for about 2 min for resumption of normal breathing. Instillations were given twice a week for 3 weeks for a total of six instillations. Mice were weighed before each i.n. administration for weight gain studies.

Blood samples for antibody measurements were collected from the tail vein of each mouse before the experiment and after three i.n. instillations of spores. Four hours after the last administration, the mice were exsanguinated and a blood sample was taken by heart puncture. Serum was separated by centrifugation for antibody measurements. An EDTA sample (Capiject T-Q Na_2-EDTA, 0.78 mg; Terumo Medical Corporation, Elkton, U.S.A.) was taken for hematological studies (35 mice total).

Hematology. Mean cell hemoglobin (MCH) and hemoglobin concentration (MCHC), mean cell volume (MCV), white blood cell count (WBC), red blood cell count (RBC), platelet count (PLT), hemoglobin (Hb), and hematocrit (HCR) were determined by an automated analyzer (Coulter T-850, Coulter Electronics Ltd, Luton, England). For white blood cell differential counts, 200 cells were counted on slides stained with May–Grünwald Giemsa.

Histological specimens. After the experiment, lung, thymus, spleen, and small intestine were collected from mice for further studies. Lung tissue specimens of one or two animals from each group were examined for bacterial (blood agar) and fungal (Sabouraud agar) growth, all with negative results, indicating that there was neither bacterial nor fungal infection present in the lung tissue. The tissue samples were immersed in fixative solution (4% formaldehyde–1% glutaraldehyde–PBS). After fixation, specimens were processed and embedded in paraffin, and sections were cut and stained with haematoxylin–eosin and periodic-acid shiff for light microscopy studies. Changes observed in lung tissue were graded from – to +++ (–, no inflammation; +, mild; ++, moderate; and +++, severe inflammation).

Antigen preparations. Both strains of *S. atra* (s. 29 and s. 72) were cultured in 50 ml of potato dextrose liquid (Difco, Detroit, MI) in 250-ml cell culture bottles (Nunc, Roskilde, Denmark). Liquid cultures were combined (300 ml) and centrifuged at 4000*g* Culture fluid antigen (CFAG) was prepared from culture supernatant. The supernatant was dialyzed extensively against water and glycerol water (1 1) for 4 hr and against PBS (Kurup *et al.,* 1978).

The pellet from the culture was washed several times with PBS and centrifuged at 4000*g* between washes. The pellet was then homogenized for 10 min and sonicated in ice for several minutes. The sample was centrifuged and the supernatant was used as a mycelial antigen (MAG) (Hearn and Mackenzie, 1979). Protein concentrations of antigen preparations were determined with the CBB method (Peterson, 1983). Protein concentrations of antigen preparations were 100–800 μg/ml.

Immunization of mice with nontoxic strain of S. atra. Three NMRI male mice (5 weeks old) were immunized intraperitoneally (ip) at weekly intervals with 100 μl of the CFAG or MAG antigens in PBS (30 μg protein/mouse) prepared from the nontoxic strain of *S. atra* (s. 29) mixed with an equal volume of Freund's incomplete adjuvant. The immunization schedule was continued for 6 weeks. Five days after the last immunization, the mice were exsanguinated, and blood samples were taken by heart puncture. Sera from the three mice immunized with either CFAG or MAG were pooled. The serum was used as a positive control in antibody measurements. Total protein concentrations of antisera were 36 mg/ml. Pooled serum from non-immunized mice served as a negative control.

Antibody measurements. An enzyme-linked immunosorbent assay was used to measure immunoglobulin G (IgG) antibodies to *S. atra.* Microwell plates (Nunc, Roskilde, Denmark) were coated with antigens (10 μg/ml: s. 72 CFAG, s. 72 MAG, s. 29 CFAG, or s. 29 MAG) in 0.1 M Na-carbonate-bicarbonate buffer, pH 9.6, 100 μl/well, overnight at 20–23°C. The plates were then washed with water. After that, the wells were blocked for an hour at 37°C with 150 μl/well 0.5% ovalbumin (w/v) (Grade IV; Sigma, St. Louis, MO) in water. The plates were washed with 0.05% Tween 20–phosphate-buffered saline, pH 7.2 (PBT). Tenfold serial dilutions of serum (from 1:20 to 1:20000) in 0.5% ovalbumin–PBT (sera before and after the intranasal instillation and positive control sera) were pipetted onto wells, and the plates were incubated for an hour at 37°C. The plates were washed with PBT; 100 μl of second antibody (goat horseradish peroxidase-conjugated anti-mouse IgG, Sigma) diluted 1:1000 in 0.5% ovalbumin–PBT was added to the wells, and the plates were incubated for 1 hr at 37°C. Then the plates were washed with PBT and 100 μl of substrate solution (0.2 mg/ml 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) Sigma, in 0.1 M Na_2HPO_4–0.05 M citric acid buffer, pH 4.7, and 0.02% H_2O_2) was added to each well. Absorbance was read after 30 min at 405 nm in a Multiplate reader (Labsystems Multiskan MCC/340, Helsinki, Finland).

Statistical analysis. The weight gains of the mice and hematological parameters were analyzed statistically by Statistix Version 4.0 (Analytical Software) with a two-sample *t* test. The significance level was $p < 0.05$.

TABLE 1

Hematological Parameters from Mice Receiving 1×10^5 or 1×10^3 Toxic or Nontoxic Spores of *Stachybotrys atra* Intranasally in 50 μl of Phosphate-Buffered Saline (PBS) Twice per Week for a 3-Week Period (a Total of Six Instillation) Compared to Those of Controls

Experimental groups	N[a]	PLT/μl[b]	WBC ($\times 10^3$/μl)[c]	EOS/μl[d]	RBC ($\times 10^6$/μl)[e]	Hb (g/liter)[f]	HCR[g]	LY (%)[h]
Control								
PBS	10	1031 ± 53	4.8 ± 0.2	0.3 ± 0.2	7.7 ± 0.29	137 ± 4	0.43 ± 0.02	90.9 ± 1.1
Toxic spores of *S. atra*								
1×10^5	8	952 ± 60	5.7 ± 0.8	1.4 ± 0.4*	8.83 ± 0.17*	155 ± 2*	0.49 ± 0.01*	90.9 ± 1.5
1×10^3	3	470 ± 99*	4.4 ± 1	2.8 ± 1.3	8.73 ± 0.23	154 ± 2	0.51 ± 0.01*	91.6 ± 1.8
Nontoxic spores of *S. atra*								
1×10^5	7	884 ± 199	5.5 ± 1	1.1 ± 0.3*	8.87 ± 0.1*	154 ± 3*	0.5 ± 0.01*	92.8 ± 1.6
1×10^3	7	834 ± 128	6.3 ± 0.8	1.3 ± 0.4*	9.1 ± 0.14*	161 ± 2*	0.49 ± 0.01*	94.8 ± 0.6*

Note. Numbers are means ± SE.

[a] Number of samples.
[b] Platelet count.
[c] White blood cell count.
[d] Eosinophil count.
[e] Red blood cell count.
[f] Hemoglobin concentration.
[g] Hematocrit.
[h] Lymphocyte %.
* Significantly different from control ($p < 0.05$).

RESULTS

Clinical Findings

One mouse (female) died during the experiment immediately after the second i.n. administration with 1×10^5 of nontoxic (s. 29) spores. This was due to too deep narcosis or too rapid intranasal administration of the slimy spore suspension which caused suffocation. No clinical signs were observed in other exposed mice. Significant differences in weight gain were not observed in exposed mice as compared to controls.

Hematology

No statistically significant differences from values for the controls receiving 50 μl PBS were detected for MHC, MCHC, or MCV in mice administrated i.n. with *S. atra* spores of either of the fungal strains or at any spore concentration. Hematological parameters with statistically significant differences are presented in Table 1. The platelet count was slightly lower in i.n. administrated mice than in controls. Hemoglobin concentration; hematocrit; and erythrocyte, eosinophil, and lymphocyte counts, were slightly higher in mice administrated i.n. with *S. atra* spores than in controls. The leucocyte count usually was higher in mice treated with spores. However, when mice were treated six times with 1×10^3 toxic spores (s. 72) the leucocyte count was lower than that in control mice. In the latter group, samples from only three mice were analyzed for hematological parameters.

Antibody Formation

I.p.-immunized mice developed IgG antibodies to *S. atra* (OD 1.0 in serum diution 1:10000 after 30 min), whereas no antibodies to *S. atra* were detected in serum in mice instilled i.n. with *S. atra* spores.

Histopathological Changes

No histopathological changes were detected in thymus, spleen, or intestines, the target organs of trichothecene mycotoxins, in any of the groups of mice.

All mice receiving 1×10^5 spores of either strain of *S. atra* or 1×10^3 spores of the toxic strain (s. 72) showed inflammatory changes in the lungs, whereas the lungs of animals receiving 1×10^3 spores of nontoxic (s. 29) *S. atra* strain or PBS remained normal. There was a significant difference in the severity of histological changes between mice instilled i.n. with the spores of toxic *S. atra* and spores of the nontoxic strain. Mice receiving 1×10^5 spores of toxic *S. atra* (s. 72) developed severe inflammatory changes (graded from ++ to +++ in all lung specimens) within both bronchioles and adjacent alveoli (Fig. 1); this was characterized by neutrophils, macrophages, and lymphocytes associated with numerous spores of *S. atra*. Interstitial inflammation with hemorrhagic exudate in the alveolar lumina was also present. Bronchiolar obliteration by inflammatory cells associated with spores of the fungus could also be detected (Fig. 1). In mice receiving 1×10^5 nontoxic spores,

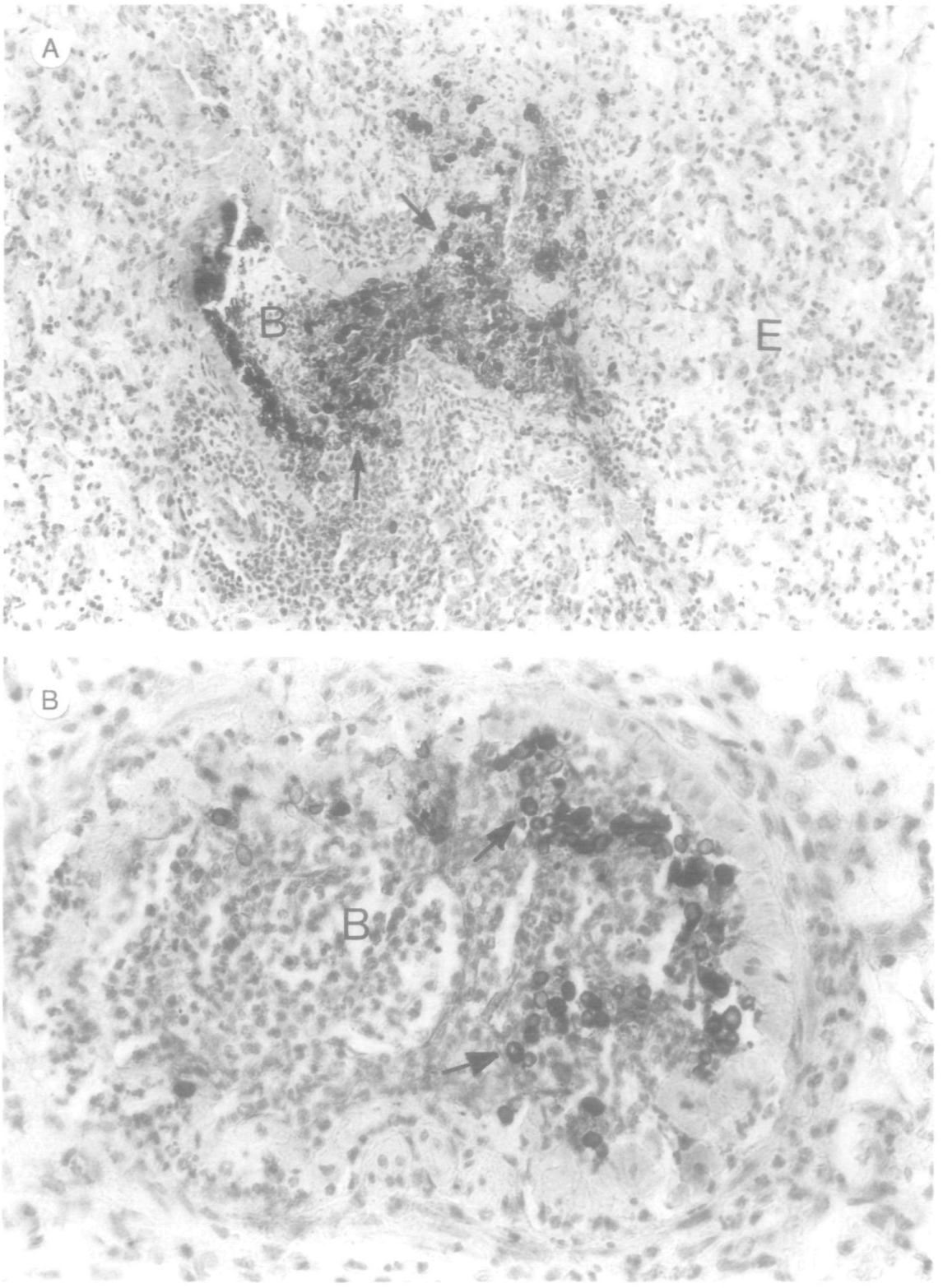

FIG. 1. Lung tissue from a mouse receiving 1×10^5 spores of toxic *S. atra* (s. 72) for a 3-week period. (A) Inflammatory changes can be seen both within bronchioles and alveoli. Hemorrhagic exudate (E) is present in alveoli. Note the obliteration of the bronchiolar lumen (B) with inflammatory cells and fungal spores (arrows) (HE, ×40). (B) Higher magnification. Obliteration of a bronchiole (B) with aggregation of inflammatory cells and spores (arrows) of *S. atra* (HE, ×100).

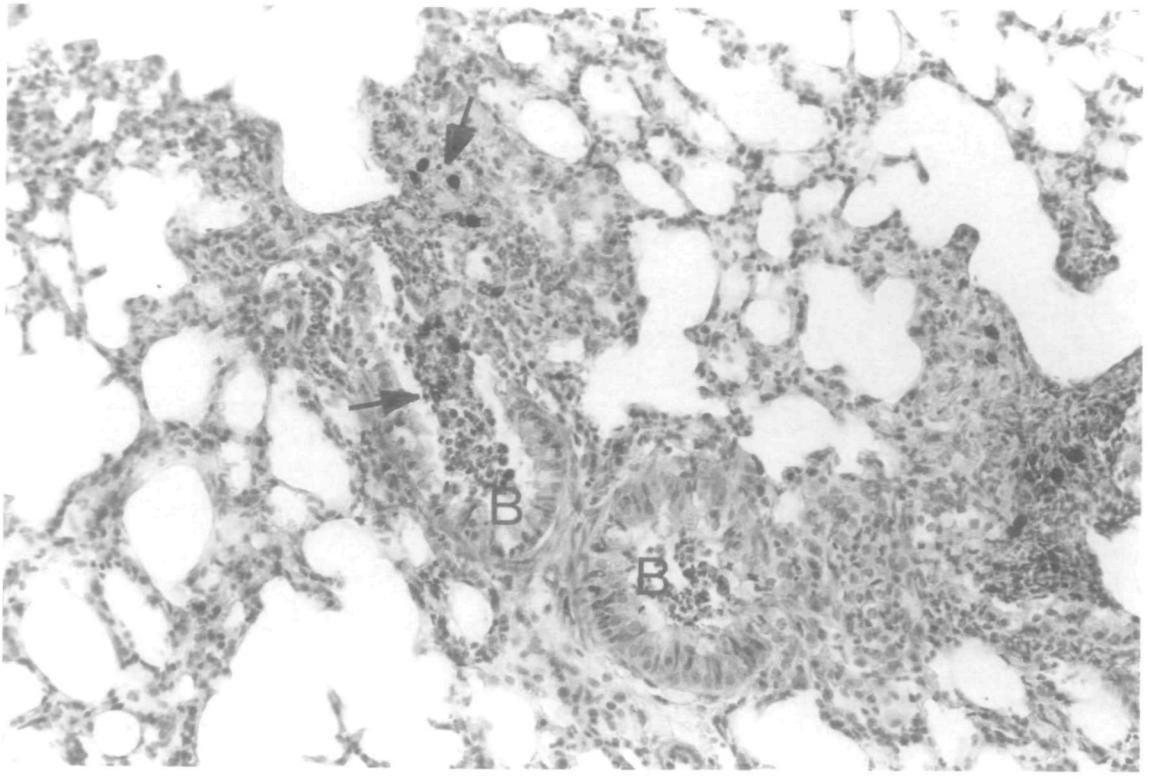

FIG. 2. Lung tissue from a mouse receiving 1×10^3 spores (arrows) of toxic *S. atra* (s. 72) for a 3-week period. Inflammation within and around bronchioles (B) can be seen with neutrophilic granulocytes, macrophages, and lymphocytes associated with spores (arrows) (HE, ×40).

inflammatory changes were similar but much milder than those in mice injected with an equal number of toxic spores. In these mice, inflammatory changes varied from + to ++. Administration with 1×10^3 spores of *S. atra* (s. 72) (Fig. 2) resulted in similar but less severe inflammation than that detected in mice instilled with 1×10^5 spores of s. 72. Inflammation in tissue samples varied from + to ++. Therefore, the inflammatory changes in the lungs were as severe in mice instilled with 1×10^3 spores of s. 72 (toxic) and 1×10^5 spores of s. 29 (nontoxic). In mice receiving 1×10^3 spores of s. 29, inflammatory changes could not be detected in the lungs.

DISCUSSION

In the present study, a murine model was developed in which mice received by intranasal instillation spores of a highly toxic or nontoxic strain of *S. atra* into their lungs.

Ueno (1984) has reported on the inhalation toxicity of T-2 mycotoxin in mice, with inhalation of T-2 toxin in solution or a culture filtrate of *Fusarium sporotrichioides* containing T-2 toxin causing death. In other animal experiments with aerosolized T-2 toxin, the test aerosols were generated from ethanol solutions of T-2 toxin with a nebulizer, and toxin was administered to the animals by nose-only exposure in chambers or by a tracheal tube. In these inhalation experiments only minor or no changes in the respiratory tract were observed after T-2 toxin exposure (Creasia *et al.*, 1987, 1990; Pang *et al.*, 1987; Thurman *et al.*, 1988). However, the histological changes in spleen, thymus, and intestines (cell necrosis and lysis) have been similar to those observed when T-2 toxin was given parenterally. Additionally, the severity of tissue damage has been dependent on the inhaled dose of T-2 toxin. Creasia and Thurman (1993) hypothesized that ethanol, the T-2 aerosol vehicle used in the previous studies, might have enhanced the translocation of T-2 toxin from the respiratory tract to other organs, which thus could have produced systemic rather than local respiratory toxicity. They examined the effects of PBS as the aerosol vehicle for T-2 toxin in mice. The administration of the toxin in PBS did not, however, produce any inflammatory changes in the respiratory tract.

In the present study, fungal spores in PBS served as carriers of mycotoxins in order to simulate the situation in which animals or humans inhale fungal propagules. In addition to the mycotoxins, fungal propagules contain allergens and biologically active cell wall components (e.g., (1–3)-β-D-glucan) (Horner *et al.*, 1994; Williams, 1994). In the present study, the highly toxic strain of *S. atra* (s. 72) produced spores with high concentrations of trichothecene mycotox-

ins, satratoxins G and H, and compounds of the group of phenylspirodrimanes, stachybotrylactone and -lactam. The nontoxic spores (s. 29) contained only minor amounts of phenylspirodrimanes, the anticomplement, and immunosuppressant compounds produced by *Stachybotrys* spp. (Ayer and Miao, 1993; Miyazaki *et al.*, 1980).

In the study of acute inhalation toxicity of T-2 toxin in mice, the LD50 values were 0.24 mg/kg body wt for young adult mice and 0.94 mg/kg body wt for mature mice (Creasia *et al.*, 1987). In the present study, when 1×10^5 toxic *S. atra* spores were injected intranasally six times, approximate concentrations of satratoxins G and H, spirolactone, and spirolactam were 1 μg/kg, 2.6 μg/kg, 2 mg/kg, and 0.5 mg/kg body wt.

In animals with stachybotryotoxicosis, the hematopoietic system is affected, resulting in a decrease in leucocyte and platelet counts (Száthmary, 1983). In inhalation exposure to T-2 toxin in pigs, hematological alterations included a decrease in hemoglobin and lymphocyte counts and an increase in neutrophil counts (Pang *et al.*, 1988). In the present study, after intranasal administration of both strains of *S. atra* and at both spore concentrations, minor changes in hematological parameters were detected in the mice. Such hematological changes could be due to both mycotoxins and other compounds from fungal propagules. The differences were usually statistically significant ($p < 0.05$).

In an epidemiological and immunochemical study of an office building with moisture problems, material samples were found to be contaminated with *S. atra* and contained satratoxin H and phenylspirodrimanes. Exposed employees showed abnormalities in their cellular and humoral immunity. However, the magnitude of these changes was small. IgG antibodies to *S. atra* in these employees did not differ significantly from those of controls (Johanning *et al.*, 1996). Elevation of IgG antibodies in serum indicates an exposure to relevant antigens. On the other hand, mycotoxins can reduce this elevation. In an earlier study, the release of IgG into the culture medium by a mouse IgG-producing myeloma cell line (MOPC 31/C) was inhibited with 5 ng/ml of T-2 toxin (Otokawa, 1983). In the present study, IgG antibodies to *S. atra* could not be detected in sera of mice exposed to *S. atra* spores intranasally.

Histological changes in the present study differ from findings in animal studies involving aerosolized T-2 toxin (Creasia *et al.*, 1987; 1990; Creasia and Thurman, 1993; Marrs *et al.*, 1986; Pang *et al.*, 1987; Thurman *et al.*, 1988). No histological changes were observed in the present study in thymus, spleen, or intestines, those organs where tissue damage has earlier been detected after exposure to T-2 toxin; in fact, intranasal exposure to both toxic and nontoxic spores of *S. atra* caused inflammation in the lung tissue, with the most severe reaction occurring within the bronchioles and surrounding alveoli. In studies of hypersensitivity pneumonitis, which is a cell-mediated allergic alveolitis, inflammation in bronchioles and alveoli has also been detected; however, fragments of fungi or spores have only rarely been detected in lung biopsies from patients with "farmer's lung" (Reijula and Sutinen, 1985).

In the present study, the severity of changes in lung tissue was clearly dependent on toxicity of spores and on spore concentration. Spores ($1 \times 10^5 \times 6$ for 3 weeks) containing satratoxins caused severe inflammatory changes in the lung tissue with hemorrhagic exudate in the alveolar lumina, and $1 \times 10^3 \times 6$ toxic spores caused a similar but less severe inflammatory reaction. The significantly milder inflammatory reaction seen in the lung tissue of the mice treated with $1 \times 10^5 \times 6$ nontoxic spores could be due to the small amounts of spirolactones and -lactams, or to the large number of spores instilled into the lungs, or to both. Inflammatory reactions or tissue changes were not seen in lung tissue in mice exposed intranasally to $1 \times 10^3 \times 6$ nontoxic spores of *S. atra*. The systemic changes seen in the mice can be due to all compounds from fungal propagules.

In an earlier experiment, necrosis was detected in the lungs of mice exposed intranasally to a high number of toxic spores of *S. atra* in areas where spores were also present (Nikulin *et al.*, in press). In the present study, the absence of necrosis in the lung suggests that the concentration of mycotoxins was so low that necrosis failed to develop.

The present study suggests that intranasal exposure to fungal spores carrying cytotoxic trichothecene mycotoxins (satratoxins) produces severe inflammation in the lungs, and that the severity of this inflammation is dependent on the toxicity of spores and on spore concentration. The response in the lungs was significantly different from the response due to inhalation exposure to aerosolized particles of structurally satratoxin-like T-2 toxin alone. It seems that spores themselves may play an important role in the responses detected in lungs of animals exposed intranasally to such spores.

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