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Experimental lung mycotoxicosis in mice induced by *Stachybotrys atra*

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Summary. *Stachybotrys atra* is often isolated from building materials in houses with moisture problems. Spores of *S. atra* can contain mycotoxins which may lead to various symptoms in exposed residents in damp houses. The pathogenesis of *S. atra*-induced lung diseases has not been elucidated. The purpose of the present study was to investigate lung mycotoxicosis experimentally in mice after an intranasal exposure to spores of *S. atra*-fungus. One group of mice received one intranasal injection of spores of a toxic strain of *S. atra* (1×10^6 spores) and the other group spores of a less toxic strain. Spores of both strains contained spirolactones and spirolactams while the highly toxic strain contained also trichothecene mycotoxins, satratoxins. The spores containing satratoxins caused severe intra-alveolar, bronchiolar and interstitial inflammation with haemorrhagic exudative processes in the alveolar and bronchiolar lumen. A significant difference was observed in the severity of the lung damage caused by the two strains of *S. atra*. The spores without satratoxins induced a milder inflammation, so that the toxic compounds of *S. atra*-spores are most likely responsible for the severity of the lung injury.

Keywords: Stachybotrys atra, pulmonary mycotoxicosis, satratoxins, intranasal exposure

The fungus *Stachybotrys atra* (synonym of *S. chartarum*) can cause the severe intoxication, stachybotryotoxicosis, in animals after ingestion of fodder contaminated by *S. atra.* Persons handling *Stachybotrys*-contaminated fodder have suffered from cough, rhinitis, burning sensation in the mouth and nasal passages, and cutaneous irritation due to the toxin contact (Hintikka, 1978, Szathmary, 1983). Highly toxic macrocyclic trichothecenes and other compounds have been isolated from cultures of toxic *S. atra*-strains (Jarvis, 1991, Jarvis *et al.*, 1995).

In recent years *S. atra* 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.*, 1993, Miller *et al.*, 1988). In buildings contaminated with *Stachybotrys*, residents have suffered from cough, irritation of eyes, skin and respiratory tract, headache and fatigue. These symptoms

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have also been experienced in human stachybotryotoxicosis after inhalation of or direct contact with *Stachybotrys*-fungi (Croft *et al*., 1986, Johanning *et al*., 1993). Spores of *S. atra* can contain toxins (Sorenson *et al*., 1987). Futhermore, *S. atra* is able to grow and to produce toxins on building materials, e.g. wallpaper and gypsum board covered with cardboard (Nikulin *et al*., 1994). No experimental inhalation studies with *S. atra*-spores have thus far been published to demonstrate their possible effects on respiratory tract and lung tissue.

The aim of the present experimental study in mice was to investigate the early response of lung tissue after exposure to inhaled *S. atra* spores.

Methods

Stachybotrys atra strains

Two strains of *S. atra*, one slightly toxic (s. 29) and one highly toxic (s. 72), were grown on rice flour (7.5%) agar (2%) for two weeks at 20–23°C and for 2 weeks at 6–8°C.

Toxicity of spores

Spores from agar plates were suspended in phosphate-buffered saline (PBS), pH 7.2, and counted, and 1×10^8 spores were centrifuged (20 min, 1300*g*). The supernatant was discarded, the spores were dried overnight (50°C), and toxins from the spores were extracted with 95% methanol by sonication for 10 minutes to release the toxins into the methanol. The extract was run through a filter paper. The methanol fraction was then divided into two equal parts and the methanol was evaporated in a nitrogen atmosphere. One fraction was diluted in 10% methanol-PBS (1 mg dried crude extract/ml); the toxicity of this sample was tested on a feline fetus lung cell line as described previously (Pasanen *et al*., 1993). After 5 days' incubation, 50% inhibition of cell proliferation was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) bioassay as described by Dombrink-Kurtzman and co-workers (1994).

The other fraction was dissolved in 100 μl of methanol and 5 μl was injected into a high performance liquid chromatograph (HPLC) with a Supelco LC-18 (5 μm), 250 × 4.6 mm column, flow rate 1.2 ml/min and UV-detector (260 nm). A water gradient containing 5% acetic acid in water (A) and methanol (B) was used: from time (*t*) 0 to *t* 3.15 min 40% A, 60% B, *t* 3.15 min to *t* 8 min gradient of 60–80% B, *t* 8 min to *t* 12 min 80% B, *t* 12 min to *t* 13 min gradient of 80–90% B and *t* 13 to *t* 20 min 90% B.

Intranasal injection of S. atr *spores*

Three groups of four NMRI mice (National Veterinary and Food Research Institute, Helsinki, Finland) aged 5 weeks, weighing 21.2 ± 1.4 g were used. The principles of laboratory animal care formulated by the Faculty of Veterinary Medicine, University of Helsinki, Finland, were followed during the experiment. A spore suspension of 1×10^6 *S. atra* spores in 50 μl of PBS was sonicated for 20–30 s to create a single spore suspension. PBS samples (filtered through a 0.2-μm filter) were taken from both of the strains for the cytotoxicity test. Because the PBS sample from the s. 29 strain was not cytotoxic, and the PBS sample from the s. 72 strain was only slightly toxic to cells, it can be supposed that the toxins are mainly in spores in a form only slightly soluble in PBS, and spores act as the carrier vehicle of toxins.

The mice were slightly anaesthetized with ether before intranasal injection of 1×10^6 spores of *S. atra* s. 29 or s. 72 in 50 μl of PBS. The first group received one injection of the s. 72, and the other group one injection of the s. 29 fungal strain; the third group received 50 μl of PBS (control group). The mice were weighed before and after the 3-day experiment.

Histological specimens

The mice were exsanguinated 3 days after the intranasal injection. Excised lung tissue specimens were prepared by gently introducing fixative solution (4% formaldehyde–1% glutaraldehyde–PBS) into the right lung with a syringe and needle inserted into the trachea. A piece of lung was then immersed in the fixative solution and kept at 4 °C overnight. After inflation fixation, specimens were taken for paraffin embedding; sections were cut at 5 μm and stained with haematoxylin-eosin and periodic acid-Schiff (PAS).

Results

Methanol-extractable solid from the strain s. 29 proved to be slightly toxic (0.8 mg crude extract/ml caused 50% inhibition of cell growth) on feline fetus lung cells, whereas the strain s. 72 was extremely toxic (60 ng crude extract/ml caused 50% inhibition of cell growth). The toxic *S. atra* strain (s. 72) showed about 13 000-fold more toxicity to the cells than did the slightly toxic strain (s. 29).

The HPLC analysis showed that satratoxins G and H (0.04 μg and 0.1 μg/l × 10^6 spores) and stachybotrylactone and stachybotrylactam (80 μg and 20 μg/l × 10^6 spores) were present in the culture of *S. atra* s. 72. No

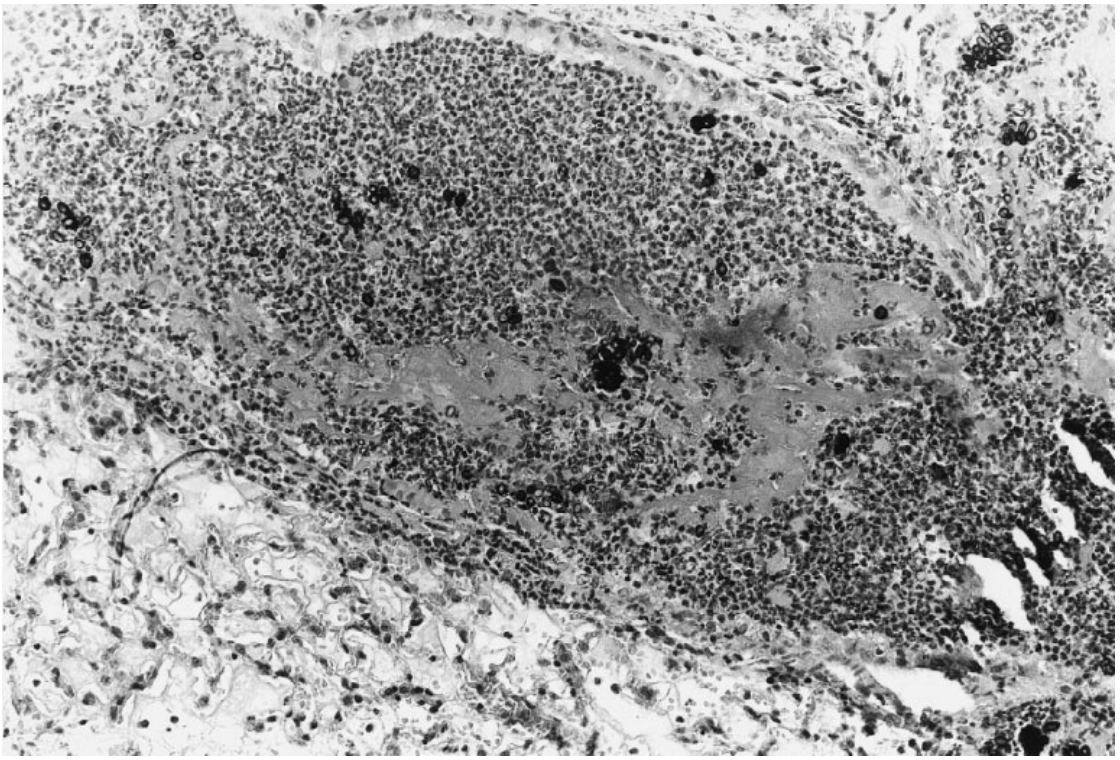

Figure 1. Histology of mouse lung tissue after intranasal injection of toxic *S. atra* strain (s. 72): severe intrabronchiolar inflammation with numerous neutrophilic granulocytes and macrophages. Note fungal spores and necrotic area in the centre of the bronchiolar lumen. In the intra-alveolar space oedematous fluid with some macrophages and granulocytes is apparent. HE, × 100.

satratoxins and only minor amounts of stachybotrylactone and stachybotrylactam were detected in the s. 29 culture.

The four mice exposed intranasally to spores of the less toxic strain (s. 29) showed no clinical symptoms. The four mice exposed to spores of the toxic strain (s. 72) became lethargic after exposure; one of these died 10 hours after dosing, and one was exsanguinated in a moribund state 24 hours after dosing. The remaining two mice survived the 3-day observation period. The weight of all 12 mice averaged 21.2 ± 1.4 g before the experiment. Control mice and those treated with *S. atra* s. 29 weighed 23.5 ± 1.6 g after the 3-day observation period, while *S. atra* s. 72-treated mice weighed 17.7 ± 0.1 g after the exposure.

All mice receiving *S. atra* spores developed inflammatory lung lesions observable histologically, while those injected with PBS remained normal. There was, however, a significant difference in the inflammatory changes produced in mice receiving spores of *S. atra* strain s. 29 and those receiving s. 72. Spores of strain s. 72 induced severe intra-alveolar and interstitial inflammation with haemorrhagic exudate in alveolar lumina (Figure 1). Further, intra-alveolar and intrabronchiolar obliteration with focal aggregation of inflammatory cells, mainly neutrophils and macrophages, occurred in s. 72-exposed mice. In addition, neutrophilic granulocytes and macrophages with fungal spores were found in the lung parenchyma. Some lymphocytes were found in the interstitium. The inflammatory cells and fungal material were forming focal aggregations most often located in the peribronchiolar area. Necrotic changes occurred in the centres of the focal areas of inflammatory cells and spores (Figure 2). These histopathological changes were the same in the mice which died before the end of the experiment and in those exsanguinated after 3 days of exposure. The inflammatory response after s. 29 exposure was significantly milder than that produced by s. 72, and no necrotic changes were detected.

Discussion

Toxigenic *S. atra* cultures produce satratoxins (Bata *et al.*, 1985, Harrach *et al.*, 1981, 1983, 1987), including some of the most toxic macrocylic 12,13-epoxy-9-trichothecenes. Ingested trichothecenes have caused

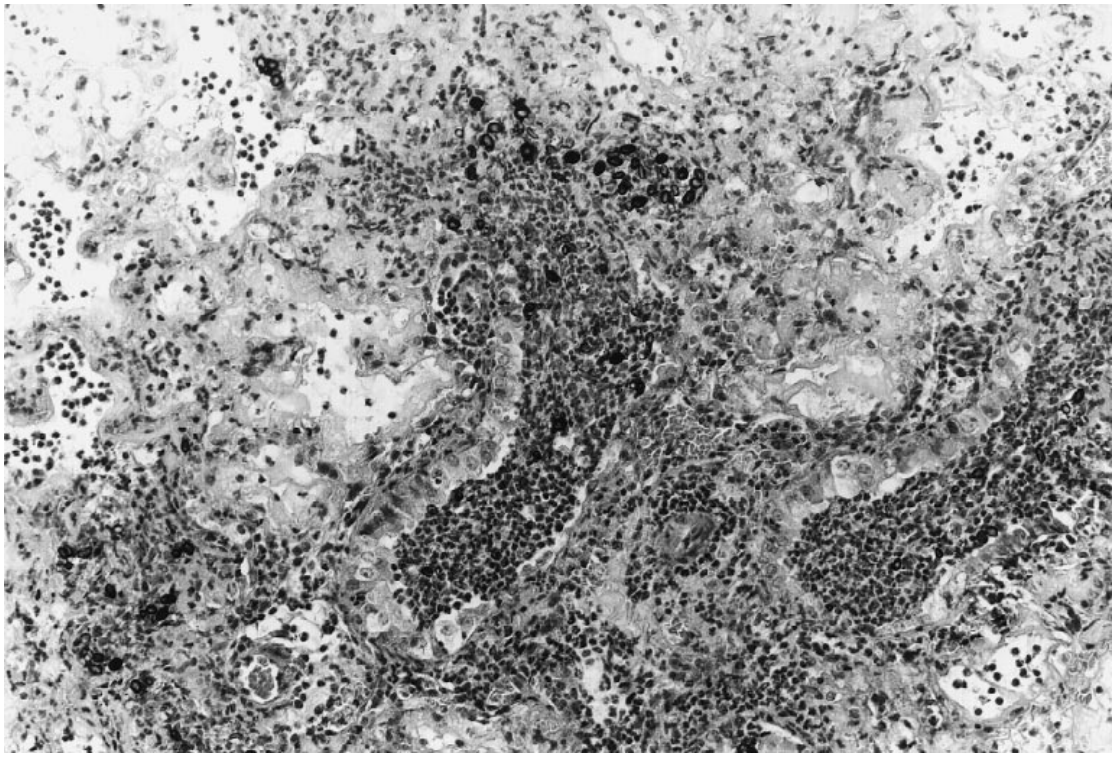

Figure 2. Mouse lung after intranasal injection of *S. atra* (s. 72). Intrabronchiolar inflammation with granulocytes and macrophages visible. Note fungal spores in bronchiolar and alveolar spaces, as well as oedema and an increased number of inflammatory cells. HE, × 100.

weight loss and death in animals both in field outbreaks and laboratory experiments. Trichothecenes are highly irritant to skin and cause severe lymphocyte depletion and necrosis in both the B-cell and T-cell-dependent regions of the lymphoid organs (WHO, 1990). In animals, haemorrhagic syndrome is a characteristic feature of ingestion of mouldy fodder contaminated by *S. atra*. (Schneider *et al.*, 1979, Szathmary, 1983). Purified trichothecenes and *S. atra* extracts are able to cause lysis of erythrocytes (DeLoach *et al.*, 1989, Hintikka, 1977, Rizzo *et al.*, 1992).

In the present study, the spores of the toxic s. 72 strain of *S. atra* contained satratoxins and large quantities of stachybotrylactone and stachybotrylactam. Spirolactones and lactams act as anticomplement compounds (Miyazaki *et al.*, 1980). The death of two mice during the first 24 h of the experiment confirmed the toxicity of the spores of the s. 72 strain, as did the weight loss of mice injected intranasally with spores of the toxic strain, although no weight loss was observed in mice exposed to spores of the considerably less toxic strain.

In *Aspergillus fumigatus*-induced lung disease the presence of eosinophils and granuloma formation has been detected (Kurup *et al.*, 1990), but only rarely have spores of fungi been demonstrated in the lung parenchyma of human hypersensitivity pneumonitis. In farmer's lung where the aetiological agent was *A. fumigatus*, fragments of fungi were detected by immunohistology and electronmicroscopy (Reijula & Sutinen, 1985, 1986). In the present animal model, spores of *S. atra* were clearly visible even without immunostaining. The aggregations of inflammatory cells which often contained fungal spores were detected both in the intra-alveolar and bronchiolar space and peribronchiolar areas.

Creasia and co-workers (1987) studied in mice the acute inhalation toxicity of pure T-2 toxin, a trichothecene mycotoxin produced by various species of *Fusarium* fungi. After the exposure of mice to aerosols of T-2 toxin no significant pathological changes were detected in either the upper respiratory tract or lungs. In the present study, spores of the highly toxic (s. 72) and less toxic (s. 29) *S. atra* strains served as carriers of the toxins in intranasal injection. The necrosis in the lung cells seen only after the s. 72 injection was obviously due to toxic substances in the spores. In hypersensitivity pneumonitis, lymphocyte and plasma cells predominate in lung tissue (Sutinen *et al.*, 1985). In the present animal model, neutrophilic granulocytes and macropahges were

the most common cells found in the inflammatory process. However, some lympocytes were also present. Furthermore, haemorrhagic inflammatory lung injury occurred in the present experiment when mice were exposed to spores of the strain of *S. atra* containing trichothecene mycotoxins, satratoxins. Thus, the present findings clearly suggest that spores carrying toxins produce a response in lungs different from the reaction due to exposure to purified toxins alone.

Case histories have been described of 23 people developing severe respiratory symptoms, including breathing difficulties, dyspnoea and sore throat after handling straw heavily contaminated with *Stachybotrys*. The authors suggested that the cause of symptoms was inhalation of toxic spores of *Stachybotrys* (Andrassy *et al*., 1979). *Stachybotrys atra* has occasionally been isolated from air samples collected in buidings with mould problems. Up to 18 000 colony forming units/m^3 have been detected by use of the Anderssen air sampler (Hunter *et al*., 1988). However, spore counts as high as this have rarely been detected, because *Stachybotrys* produces spores in a slimy mass and they become airborne only when they dry.

In the present study, both toxic and less toxic strains of *S. atra* caused an inflammatory reaction in the mouse lung, but the more toxic strain caused a much more severe reaction (e.g. cell necrosis and haemorrhage). Such inflammatory reactions caused by *Stachybotrys* have not been described in humans. Emanuel and co-workers (1975) described pulmonary mycotoxicosis in farm workers who inhaled massive quantities of airborne dust when removing mouldy silage. A fungus capable of producing trichothecene mycotoxins (*Fusarium* sp.) was isolated from a lung biopsy of one patient, which suggested that toxicity may play a major role in the inflammatory reaction and obliterative bronchiolitis in the lung biopsy of the patient. In the present study, the spore count of *Stachybotrys* was high. Comparing the present experiment to inhalation exposure in humans, they are unlikely to be exposed to spore counts so high even in very mouldy environments. However, it might be possible for minor amounts of *Stachybotrys* spores to cause inflammatory reactions in humans, with severity of reactions obviously dependent on the toxicity of the spores. It is also nesessary to carry out dose– response studies for risk assessment in humans.

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References

Andrassy K., Horvath J., Lakos T. & Töke Zs. (1979) Mass incidence of mycotoxicosis in Hajdu-Bihar county. *Mykosen* **23,** 130–133. In German. Abstract in English.

Bata A., Harrach B., Ujszaszki K., Kis-tamas A. & Lasztity R. (1985) Macrocylic trichothecene toxins produced by *Stachybotrys atra* strains isolated in Middle Europe. *Appl. Environ. Microbiol.* **49,** 678–681.

Bisset J. (1987) Fungi associated with urea-formaldehyde insulation in Canada. *Mycopathol.* **99,** 47–56.

Creasia D.A., Thurman J.D., Jones III L.J., Nealley M.L., York C.G., Wannemacher Jr R.W. & Bunner D.L. (1987) Acute inhalation toxicity of T-2 mycotoxin in mice. *Fundam. Appl. Toxicol.* **8,** 230–235.

Croft W.A., Jarvis B.B. & Yatawara C.S. (1986) Airborne outbreak of trichothecene toxicosis. *Atm. Environ.* **20,** 549–552.

Deloach J.R., Gyongyossy-issa M.I.C. & Khachatourians G.G. (1989) Species-specific hemolysis of erythrocytes by T-2 toxin. *Toxicol. Appl. Pharmacol.* **97,** 107–112.

Dombrink-kurtzman M.A., Bennet G.A. & Richard J.L. (1994) An optimized MTT bioassay for determination of cytotoxicity of fumonisins in turkey lymphocytes. *J. AOAC Int.* **77,** 512–516.

Emanuel D.A., Wenzel F.J. & Lawton B.R. (1975) Pulmonary mycotoxicosis. *Chest* **67,** 293–297.

Harrach B., Bata A., Bajmocy E. & Benko M. (1983) Isolation of satratoxins from the bedding straw of a sheep flock with fatal stachybotryotoxicosis. *Appl. Environ. Microbiol.* **45,** 1419–1422.

Harrach B., Bata A., Sandor G. & Vanyi A. (1987) Isolation of macrocylic and non-macrocylic trichothecenes (*Stachybotrys* and *Fusarium* toxins) from the environment of 200 ill sport horses. *Mycot. Res.* **3,** 65–68.

Harrach B., Mirocha C.J., Pathre S.V. & Palyusik M. (1981) Macrocylic trichothecene toxins produced by a strain of *Stachybotrys atra* from Hungary. *Appl. Environ. Microbiol.* **41,** 1428–1432.

Hintikka E.-L. (1977) The genus *Stachybotrys*. In *Mycotoxic fungi. Mycotoxins. Mycotoxicoses*. Eds T.D. Wyllie & L.G. Morehouse, Vol. 1. New York: Marcel Deccer Inc, pp. 91–98.

Hintikka E.-L. (1978) Human stachybotryotoxicosis. In *Mycotoxic fungi. Mycotoxins. Mycotoxicoses*. Eds T.D. Wyllie & L.G. Morehouse, Vol. 3. New York: Marcel Deccer Inc, pp. 87–89.

Hunter C.A., Grant C., Flannigan B. & Bravery A.F. (1988) Mould in buildings: the air spora of domestic dwellings. *Int. Biodeter.* **24,** 81–101.

Jarvis B.B. (1991) Macrocylic trichothecenes. In *Mycotoxins and Phytoalexins*. Eds R.P. Sharma & D.K. Salunkhe. Boca Raton: CRC Press, pp. 361–421.

Jarvis B.B., Salemme J. & Morais A. (1995) *Stachybotrys* toxins. 1. *Nat. Tox.* **3,** 10–16.

Johanning E., Morey P.R. & Goldberg M. (1993) Remedial techniques and medical surveillance program for the handling of toxigenic *Stachybotrys atra*. In *Proceedings of the 6th International Conference on Indoor Air Quality and Climate* Eds P.

Kalliokoski, M. Jantunen & O. Seppänen, Vol. 4. Jyväskylä, Finland: Gummerrus Oy, pp. 311–316.

KURUP V., CHOI H., RESNICK A., KALBFLEISCH J. & FINK J. (1990) Immunopathological response of C57BL/6 and C3H/HeN mice to *Aspergillus fumigatus* antigens. *Int. Arch. Allergy Appl. Immunol.* **91,** 145–154.

MILLER J.D., LAFLAMME A.M., SOBOL Y., LAFONTAINE P. & GREENHALGH R. (1988) Fungi and fungal products in some Canadian houses. *Int. Biodeter.* **24,** 103–120.

MIYAZAKI W., TAMAOKA H., SHINOHARA M., KAISE H., IZAWA T., NAKANO Y., KINOSHITA T., HONG K. & INOUE K. (1980) A complement inhibitor produced by *Stachybotrys complementi*, nov. sp. K-76, a new species of fungi imperfecti. *Microbiol. Immunol.* **24,** 1091–1108.

NIKULIN M., PASANEN A.-L., BERG S. & HINTIKKA E.-L. (1994) *Stachybotrys atra* growth and toxin production in some building materials and fodder under different relative humidities. *Appl. Environ. Microbiol.* **60,** 3421–3424.

PASANEN A.-L., NIKULIN M., TUOMAINEN M., BERG S., PARIKKA P. & HINTIKKA E.-L. (1993) Laboratory experiments on membrane filter sampling of airborne mycotoxins produced by *Stachybotrys atra* Corda. *Atm. Environ.* 27 A, 9–13.

REIJULA K. & SUTINEN S. (1985) Detection of antigens in lung biopsies by immunoperoxidase staining in extrinsic allergic bronchiolo-alveolitis (EABA). *Acta Histochemica* **75,** 121–125.

REIJULA K. & SUTINEN S. (1986) Ultrastructure of extrinsic allergic bronchiolo-alveolitis. *Path. Res. Pract.* **181,** 418–429.

RIZZO A.F., ATROSHI F., HIRVI T. & SALONIEMI H. (1992) The hemolytic activity of deoxynivalenol and T-2 toxin. *Nat. Tox.* **1,** 106–110.

SCHNEIDER D.J., MARASAS W.F.O., DALE KUYS J.C., KRIEK N.P.J. & VAN SCHALKWYK G.C. (1979) A field outbreak of suspected stachybotryotoxicosis in sheep. *J. South African Vet. Assoc.* **50,** 73–81.

SORENSON W.G., FRAZER D.G., JARVIS B.B., SIMPSON J. & ROBINSON V.A. (1987) Trichothecene mycotoxins in aerosolized conidia of *Stachybotrys atra*. *Appl. Environ. Microbiol.* **53,** 1370–1375.

SUTINEN S., REIJULA K., HUHTI E. & KÄRKÖLÄ P. (1985) Extrinsic allergic bronchiolo-alveolitis: serology and biopsy findings. *Eur. J. Respir. Dis.* **64,** 271–282.

SZATHMARY C.I. (1983) Trichothecene toxicoses and natural occurrence in Hungary. In *Trichothecenes*. Developments in Food Science 4. Ed Y. Ueno. Amsterdam: Elsevier, pp. 229–250.

WHO. (1990) Trichothecenes. In *Selected mycotoxins: ochratoxins, trichothecenes, ergot*. International programme on chemical safety. Environmental health criteria 105. WHO, Vammala, Finland: Vammalan Kirjapaino Oy, pp. 73–164.