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Intratracheal Exposure of Rats to *Aspergillus fumigatus* Spores Isolated from Sawmills in Sweden

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Five strains of *Aspergillus fumigatus* (A, B, D, H, and K) isolated from sawmills were used to expose groups of three rats by intratracheal intubation. The dose was 10^9 spores per rat. At 48 h after administration, two rats from the D group and all rats from the K group died with symptoms of strong dyspnea and tachypnea. At 72 h postadministration and after, some animals showed mild to moderate dyspnea and tachypnea. Autopsies of all animals were performed, including a histopathological examination of the lungs. At 72 h after administration, two distinct morphological groups were identified histopathologically. Severe necrotizing pneumonia characterized by the presence of abundant fungal hyphae was seen in animals that died spontaneously within 48 h postadministration and rats with bronchopneumonia and was characterized by the presence of numerous fungal spores. There was an obvious difference in pathogenicity among the strains of *A. fumigatus*. Strains D and K were more pathogenic, and only the rats exposed to these strains showed the presence of fungal hyphae in the lungs. The mycotoxin gliotoxin that is produced by *A. fumigatus* and has antiphagocytic activity was not detected in the spores from any of the *A. fumigatus* strains.

Exposure to high doses of airborne fungal diaspores in the working environment at sawmills can cause a human pulmonary disease, wood-trimmers' disease, among wood trimmers and sorters (1, 22). Two distinct forms are seen, one acute with influenzalike symptoms (fever, shivering, cough, dyspnea, and malaise), and one manifest form with progressive dyspnea and lung fibrosis (allergic alveolitis). Although prevalence figures are not completely known, the acute form is much more common than the manifest form. In one investigation (1), 10 to 20% of the exposed workers developed acute symptoms.

The artificial drying of sawn timber at sawmills in drying kilns is a serious problem because of the sometimes favorable conditions inside the kilns for growth of thermotolerant and thermophilic fungi. The relative humidity is high at the beginning of the drying schedule, and the temperature varies between 35 and 65°C. These temperatures are rather low compared with drying schedules used for other types of wood in non-Scandinavian countries. The most common species isolated from the kilns at sawmills in Scandinavian countries are *Aspergillus fumigatus* (Fres.), *Paecilomyces variotii* (Bain.), and *Rhizopus rhizopodiformis* (Cohn) (5). When the dried contaminated wood is handled in the trimming department, large amounts of fungal diaspores can contaminate the air and induce wood-trimmers' disease among the workers. The National Board of Occupational Safety and Health in Sweden issued an ordinance (Arbetarskyddsstyrelsens Författningssamling [AFS] **1988:6**) in 1988 containing provisions on wood mold and general recommendations for the implementation of the provisions. The purpose of the rules is to prevent exposure to wood molds.

The acute symptoms (sometimes called febrile attacks) in wood-trimmers' disease also occur in environments other than sawmills where exposure to fungal diaspores or mold dust is high, for instance, in farming (2, 12) and during handling of wood chips (6, 17). The relationship between the febrile attacks and allergic alveolitis is still an open question. The term organic dust toxic syndrome is now often used to describe febrile attacks after exposure to organic dust (2).

In a previous study, we established that some isolates of *A. fumigatus* from sawmills are able to produce tremorgenic mycotoxins (8). It has also been reported that *A. fumigatus* can produce another mycotoxin, gliotoxin, with antiphagocytic activity (13). In this study, we wanted to determine whether there were any differences in pathogenicity among strains of *A. fumigatus* isolated from kilns at different sawmills and also whether the strains were able to produce gliotoxin. Earlier investigations of exposure of experimental animals (predominantly mice) to *A. fumigatus* spores by inhalation or intratracheal exposure have been made (4, 7, 16, 18, 19, 21). This report describes intratracheal exposure of rats to strains of *A. fumigatus* from different sawmills in Sweden.

MATERIALS AND METHODS

Origin and isolation of fungi. Five fungal cultures of *A. fumigatus* (A, B, D, H, and K) were chosen for the experiment. In a previous experiment, strains A, B, D, and H were found to produce tremorgenic mycotoxins (8). The *A. fumigatus* cultures were isolated from progressive kilns and identified by the criteria of Raper and Fennell as described earlier (8). All isolates differing from each other in colony characteristics were regarded as separate strains. Strains A, B, and D were recently isolated, strain H was taken from the culture collection at the Department of Forest Products, Swedish University of Agricultural Sciences, Uppsala, and strain K was a fresh isolate. Strains A and B were isolated at the same time from one sawmill. Strains D and K were isolated at two different occasions 9 months apart from another sawmill. Strain H was isolated from a third sawmill.

Medium and incubation for spore production. The fungi were cultured on wood disks (diameter 50 mm, 10 mm thick)

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sawn out from fresh Scotch pine (*Pinus sylvestris*). The disks were kept frozen (−20°C) until the experiment started. The wood disks (350 g) were put in 5-liter conical flasks together with 100 ml of tap water. The flasks were autoclaved and inoculated with 50 ml of spore suspension. The spore suspensions were prepared by adding 20 ml of sterile water and 0.1 ml of Tween 80 to malt extract agar (MEA) (2.5% malt extract, 1.5% agar) medium test tube cultures followed by shaking for 10 s. The flasks were incubated in darkness at 35°C for 4 weeks (strains A, B, D, and H) or 6 weeks (strain K).

Isolation of spores. The spores were harvested from the wooden disk cultures by adding 100 ml of sterile water and 0.1 ml of Tween 80 to each flask and shaking it for 1 min. The suspension was filtered through wet cotton to remove fragments of mycelia and wood. The loss of spores during filtration was estimated to be between 30 and 40%. A known amount of the total suspension was then removed and counted under a microscope to obtain the total number of spores, which varied between 8.0×10^9 and 2.5×10^{10} per culture. The suspension was then centrifuged, and the water was decanted. The spores were kept frozen (−20°C) until use.

Gliotoxin analysis. Gliotoxin was kindly provided by Manfred Gareis, Veterinary Faculty, University of Munich, Munich, Federal Republic of Germany. The five *A. fumigatus* strains (A, B, D, H, and K) were cultivated in a separate series on wood disks as described above for 4 weeks. The spores were harvested by adding 100 to 150 ml of sterile water and 0.1 ml of Tween 80 to each flask followed by shaking for 1 min. The spore suspensions were adjusted to 10^9 spores per strain and centrifuged. The water was decanted, and 150 ml of chloroform-methanol (9:1) was added. The spore suspensions were transferred to separatory funnels and extracted twice against water with 150 ml of chloroform-methanol (9:1). The chloroform-methanol phase was evaporated to dryness on a rotary evaporator (water bath temperature, 45°C), and the residue was dissolved in minimal amounts of chloroform. Samples and standard (1 mg/ml in chloroform) were applied to thin-layer chromatography plates (Silica Gel 60; Merck 11844; E. Merck AG, Darmstadt, Federal Republic of Germany) and run in the system toluene-ethyl acetate-formic acid (5:4:1). The R_f was 0.61. Gliotoxin was detected by spraying with *p*-anisaldehyde solution (0.5 ml of *p*-anisaldehyde, 85 ml of methanol, 10 ml of glacial acetic acid, 5 ml of concentrated sulfuric acid) and heating for 15 min at 110°C (3). The toxin was then visible as a weak brown spot. The detection limit was 1 μg.

Experimental animals. A total of 18 male Wistar rats from the breeding colony from the Institute for Medical Research and Occupational Health in Zagreb were used in the experiment. The animals were 8 to 9 weeks old and weighed 160 to 190 g. During the entire experimental procedure, the animals were maintained and housed according to Organization for European Cooperation and Development guidelines for good laboratory practice. They were fed a standard rat diet (Domžale, Laboratory Rat Food) and had free access to water. Six groups of three rats each were kept in Macralon cages under controlled conditions (light, 12 h; dark, 12 h; 21°C).

Experimental design. In Sweden (and in all other countries), occupational exposure limit values for the presence of microorganisms in the air in working environments are lacking. According to the ordinance "Occupational Exposure Limit Values" (AFS **1987**:12), issued by the National Swedish Board of Occupational Safety and Health, the level limit value (that is, a limit value for a full working day) for respirable dust is 5 mg/m^3. A rough calculation shows that the weight of 10^9 spores of *A. fumigatus* is approximately 5 mg. With this background, we decided to choose a dose of 10^9 spores per rat as a starting point for the exposure experiments.

The spores were diluted with saline solution so that each preparation contained 10^9 fungal spores per 250 μl of solution. Each rat received 250 μl of suspension deep intratracheally by intubation under ether anesthesia. The control animals received only saline solution. Before intubation, the spore suspensions were vigorously shaken (Vortex-Genie k-550-GE) to break up aggregations and chains of spores. Good individualization of spores was ensured by microscopic examination of glass smears for each spore suspension. After intubation, the animals were observed regularly for 48 h. Autopsy was performed shortly after death regardless of whether animals died spontaneously or were killed at a given time. During the autopsy, multiple lung tissue samples from each rat were collected. One sample from each animal was saved in a sterile tube and frozen at −20°C, while the rest were fixed in 10% buffered neutral Formalin. Using routine histological techniques, specimens were dehydrated and embedded in paraffin, and serial 5-μm sections were obtained from each paraffin block. The first slide from each block was stained by a routine hematoxylin-eosine procedure, the second one by Gridley's stain for fungi, and the third one by Grocott's method for fungi (GMS) (11). All slides were macroscopically studied and evaluated. The lesions of interest were photographed (EFKE, KB 14, black-and-white negative film).

Isolation of fungi from lung tissue. Small pieces of the frozen lung samples collected during the autopsy were put in petri dishes, and 5 ml of sterile water was added. The petri dishes were incubated at 35°C for 3 days. Two series of cultivations were performed. In one series, samples from animals A3, B1, D3, H1, K1, and control 1 were cultivated. The time after administration was 48 to 72 h (see Table 1). In a second series, samples from animals B3, H3, and control 3 were incubated (25 weeks after administration).

RESULTS

Clinical observations. After application of the fungal spores, all the animals appeared normal for the first 12 h and no clinical symptoms related to respiratory dysfunction were observed. At 18 h after intubation, two animals administered strain D and all animals administered strain K were sluggish. At the same time, dyspnea and tachypnea were noticed in the same animals. Those symptoms were more pronounced in rats administered strain K than in animals administered strain D.

At 48 h after spore administration, two rats administered strain D (D2, D3) and all animals administered strain K died with symptoms of heavily disturbed respiratory functions (very strong dyspnea and tachypnea). At the same time, dyspnea was observed in the third rat administered strain D (D1). All other animals including rats from the control group did not shown any clinical symptoms 48 h after administration of fungal spores.

In the treated animals which survived for 72 h after administration, the symptoms could be described as mild to moderate dyspnea and tachypnea (Table 1). No tremor reactions were noticed.

Autopsy findings. During the autopsy of all treated animals that died within the first 72 h after spore administration

TABLE 1. Reactions in rats caused by intratracheal exposure to spores of *A. fumigatus* isolated from sawmills[a]

Strain and animal group	Animal no.	Reaction(s)[b] at the following time after administration:						
		48 h	72 h	6 days	9 days	17 days	3 wk	25 wk
A	A1	s, eu						
	A2	s	d^+	d, t	d^+	d^+	d^+, eu	
	A3	s	d, t, ex					
B	B1	eu						
	B2	—	—	—	—	—	eu	
	B3	—	—	—	—	—	—	eu
D	D1	s, d^+	—	—	—	d^+	d^+, eu	
	D2	d*, t, ex						
	D3	d^+, t*, ex						
H	H1	eu						
	H2	—	s, d, t	s, d, t	d, t	d, t	d, t, eu	
	H3	—	s	s, d, t	d, t	d, t	d, t	eu
K	K1	s, d, ex						
	K2	s, d, ex						
	K3	s, d, ex						
Control	Control 1	eu						
	Control 2	—	—	—	—	—	eu	
	Control 3	—	—	—	—	—	—	eu

[a] The dose was 10^9 spores per rat in saline solution.
[b] No animals showed a reaction at 24 h. s, Sluggish; ex, spontaneous death; $^+$, mild; d, dyspnea; eu, euthanasia; *, strong; t, tachypnea; —, no symptoms.

(killed or died spontaneously), the same pattern and distribution of a similar process was seen. The differences were related only to gradation of severity.

Multiple, circular foci of consolidation ranging in diameter from 1 to 4 mm covered the pleural surface and extended into deep portions of lung parenchyma. There was evidence of edema and an occasional mild degree of emphysema. The chest cavity contained an increased amount of free fluid. In animals that died spontaneously, the lesions seemed to be necrotic and much more pronounced than in those which were killed.

Disseminated, granulomatous pneumonia, fairly uniform in distribution and in the severity of the process, was observed on autopsies of all animals killed 21 days postexposure.

Only one control animal had mild edema and hyperemia of lungs, while no pathomorphological changes were observed in the other two control animals.

Histopathological findings. According to type, distribution, and severity of lesions, as observed during histological evaluation of tissue sections taken from all experimental animals, four major diagnoses were distinguished: (i) pneumonia, necrotizing, acute, disseminated, and severe, characterized by the presence of fungal hyphae, lung (animals D2 and D3 and all rats administered strain K); (ii) bronchopneumonia, suppurative, acute, multifocal, and moderate, characterized by the presence of fungal spores, lung (animals A1, B1, H1; for animal A3, the same process was coded as severe); (iii) pneumonia, granulomatous, chronic, disseminated, and moderate, characterized by the presence of fungal spores, lung (animals A2, B2, B3, D1, H2, and H3); (iv) lung tissue within normal limits (all control animals).

The following descriptions of histopathological changes correspond to each diagnosis group.

(i) The normal architecture of the lung tissue was disrupted by a number of solid areas of basophilic hypercellularity scattered throughout the whole section, mostly located peribronchially. Those areas were embedded in well-recognizable, slightly edematous lung parenchyma. The surface proportion of consolidated and recognizable lung tissue was 1:1. On high magnification, the solid areas were composed mostly of necrotic tissue debris, placed centrally and surrounded by a sparse rim of mononuclear cell infiltrate.

Starting in the necrotized center of each area, extending into the periphery, and protruding out into the free lumina of the surrounding lung tissue structures, numerous fungal hyphae were seen. Although the organisms were quite visible on hematoxylin-eosin-stained sections (the staining affinity ranges from light basophilic to amphophilic), only the special stains for fungi revealed their entire number and fine morphologic characteristics. On sections stained by GMS and Gridley's method, myriads of fungal hyphae tightly packed together were seen. The organism was of fairly uniform thickness and length and septated, with obvious dichotomous type of branching.

(ii) Large portions of lung sections were occupied by confluent foci of basophilic hypercellularity resulting from dense inflammatory cell infiltrate. The involved tissue was less than one-half of the section surface.

On higher magnification, numerous polymorphonuclear leukocytes were seen located within alveolar spaces and free lumina of the bronchial tree. There was some degree of congestion and edema, but there was no evidence of necrosis involving alveolar septae.

The special fungal stains revealed disseminated foci containing a very high number of fungal spores concentrated mostly peribronchially. There was no evidence of the presence of fungal hyphae throughout the whole section.

(iii) Throughout the entire section, a large area of basophilic hypercellularity encompassed considerable portions of lung tissue. Those areas were composed of several foci of small confluent granulomas.

On high magnification, granulomas were formed mostly of typical macrophages with admixed epithelioid cells and a

considerable number of multinucleated giant cells (both Langhans and foreign body types were present). There was no evidence of necrosis within granulomas of adjacent lung tissue.

Special stains revealed a high number of spores distributed almost in sheet pattern throughout whole granulomas. Although vigorously searched for, fungal hyphae were not observed.

Gliotoxin analysis. No gliotoxin was detected from the spores (10^9 per strain) from any of the five *A. fumigatus* strains by thin-layer chromatography (detection limit, 1 μg).

Cultivation of lung tissue samples. In the first series (time after administration, 48 to 72 h), all samples except the one from the control animal showed growth of *A. fumigatus* (confirmed under the microscope). In the second series (time after administration, 25 weeks), no growth of *A. fumigatus* was found. Parallel samples in this series were then macerated and put on MEA plates and incubated in the same manner as the pure lung tissue samples. This cultivation was also negative.

DISCUSSION

A great difference in pathogenicity of strains D and K compared with the other strains of *A. fumigatus* was observed. Necrotizing pneumonia of such extent and severity as seen in animals D2 and D3 and all the animals administered strain K is a condition incompatible with possible reparation and continuation of life. There was a striking coincidence between the severe necrosis and the presence of fungal hyphae in the lung tissue, so the causal relationship of these two facts is postulated.

The bronchopneumonia seen in animals A1, A3, B1, and H1 was severe, possibly a fatal condition, but judged on histopathological criteria, healing might well have been expected. Intrapulmonary administration of articulated material of any type might well have induced such a process. The granulomatous pneumonia seen in animals A2, B2, B3, D1, H2, and H3 was a nonspecific chronic lesion, obviously induced by the presence of fungal spores. A morphologically indistinguishable condition might have been induced by foreign body particles and also by some chronic bacterial and fungal infections.

There are two possible explanations why no tremor was recorded in this experiment. The first is that the strains had lost the ability to produce tremorgenic mycotoxins. The second is that there were no toxins in the conidia or that the amount present was below the level required to induce tremors. Thin-layer chromatographic studies of semisynthetic-medium-grown mycelia in a special cultivation performed after the intratracheal exposure revealed that none of the strains were producing any tremorgenic mycotoxins, which indicated that the producing abilities had been lost.

There have been discussions regarding the fate of fungal spores in the lungs of experimental animals and how they are eliminated by the immune system. Earlier investigations often used laboratory mice to study the effects of *A. fumigatus* spores in the lungs. Waldorf et al. (18) found neither progressive infection nor mortality in normal mice. Histopathological findings confirmed that there was no spore germination or growth. Similarly, Schaffner et al. (16) established that healthy mice were extremely resistant to inhaled conidia. Ford and Friedman (4) noticed that none of the exposed mice died and further that the lungs rapidly disposed of spores, regardless of the pathogenic potential of the fungal isolate. Interestingly, Walzl et al. (19) found that mice could fully control the infection regardless of the degree of virulence of the *A. fumigatus* strain used. There were no differences between the two strains in the morphology of lung lesions, and also, no mortality occurred. The results cited are not in accordance with the findings in this study. It is clear that two of our strains of *A. fumigatus* (strains D and K) were more pathogenic than the others since only animals exposed to these strains showed fungal growth in lung tissue that led to spontaneous death. There are no obvious reasons why these two strains should be more pathogenic. The only significant difference between the strains used was that strain K was a fresh isolate compared with the others and that it was incubated for 6 weeks instead of 4 weeks as for the rest of the strains. It is interesting that strains D and K were isolated from the same sawmill but on different occasions. Our results showed that there were variations in behavior between strains of *A. fumigatus* after introduction of spores into the lungs. This variation indicates the importance of using several isolates and not just one in these types of studies; otherwise, the results can be of limited value because of the uncertainty about the characteristics of the single isolate compared with other strains within the species.

The pulmonary defense against spores of *A. fumigatus* has been investigated and somewhat different interpretations have been put forward. Schaffner et al. (16) identified two defense lines. The first is by pulmonary alveolar macrophages, which prevent germination and kill the spores. The second is by neutrophilic granulocytes, which are active against the hyphal form of the fungi. These lines of defense function without the involvement of a specific immune response (which is B- or T-cell dependent). Kurup (7) claimed that *A. fumigatus* spores are phagocytized by pulmonary alveolar macrophages but that these do not kill the spores to any higher degree. Interestingly, spores from *Aspergillus flavus* and *Aspergillus niger* are killed by pulmonary alveolar macrophages. Waldorf et al. (18), on the other hand, said that pulmonary defense against *A. fumigatus* spores depends on early killing by bronchoalveolar macrophages. Further, the inhibition of spore germination and killing of the spores appear to constitute separate mechanisms, which may be handled by different cell types. That neutrophilic granulocytes can kill *A. fumigatus* conidia that have been preincubated (swollen) but cannot kill resting (dormant) conidia was established by Levitz et al. (9, 10). They also found that hyphae of *A. fumigatus* are killed by neutrophilic granulocytes.

Robertson et al. (14, 15) reported that *A. fumigatus* spores produce one or more substances that readily diffuse from the spore surface and have an inhibitory effect on phagocytosis in mice and rats. Diffusates from spores of another fungus (*Penicillium ochro-chloron*) had no such effect. Similarly, Washburn et al. (20) found a water-soluble substance on the conidial surface of *A. fumigatus* which inhibited activation of the alternative complement pathway. This effect also was found from *A. flavus* but not from *A. niger*. Gliotoxin, a mycotoxin produced by *A. fumigatus*, has been found to exhibit antiphagocytic activity in mice (13).

The isolation and cultivation of fungi from lung tissue samples 48 to 72 h after administration of spores showed that the spores were still viable, but the germination was obviously inhibited except for strains D and K. It is possible that the conidia were phagocytized by pulmonary alveolar macrophages but that this event did not kill the spores, in accordance with the findings of Kurup (7) and Robertson et al. (14, 15). The pathogenic strains were able to withstand

the immune response against the conidia and also against the hyphae.

Although tremorgenic mycotoxins or gliotoxin were not found in this study, the possible effect of spore diffusates with inhibitory effects (14, 15, 20) or other secondary metabolites cannot be ruled out. These results should be highly contributory in understanding the pathogenicity and mode of action of fungi and their influence on human and animal health. We also believe that new knowledge might be helpful in the prevention of mold-induced lung diseases as an occupational health problem.

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