Tremorgenic Mycotoxins from Aspergillus fumigatus as a Possible Occupational Health Problem in Sawmills

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Received 12 May 1986/Accepted 26 December 1986

Wood-trimmers’ disease, generally called extrinsic allergic alveolitis, which affects workers in sawmills, is thought to be caused by fungal diaspores. The importance of Aspergillus fumigatus on the surface of wood dried in kilns is accentuated by its ability to produce tremorgenic mycotoxins. Eight strains of A. fumigatus from five different sawmills were isolated and cultivated on liquid media, and one of the strains was also cultivated on wood blocks. Extracts were prepared, and the tremorgenic reactions were induced by oral administration of extracts to rats. Extracts of the strain grown in liquid medium and on wood blocks induced very strong tremorgenic reactions when administered orally to rats. Four other strains induced mild tremorgenic reactions. High-performance liquid chromatography analysis revealed two tremorgenic mycotoxins, verruculogen and fumitremorgen C, in the five toxic strains. One nontoxic strain produced detectable levels of verruculogen. These results, coupled with the known resemblance of the acutely toxic phase of wood-trimmers’ disease to the symptoms produced by these tremorgens, imply that wood-trimmers’ disease and similar occupational diseases are, at least in part, mycotoxocoses.

Exposure to high concentrations of airborne fungal diaspores (>10^6/m^3 of air) can cause a human pulmonary disease usually called hypersensitive pneumonitis or extrinsic allergic alveolitis. Depending upon the origin of exposure it is commonly called farmers’ lung, malt-workers’ disease (3), wood-trimmers’ disease, etc. (1, 16, 24). Usually a distinction is made between the acute and chronic forms of the disease. The former is often called acute alveolitis, and the latter is often called allergic alveolitis. The acute reaction is characterized by fever, shivering, cough, dyspnea, and malaise 4 to 8 h after exposure. After 24 h the symptoms usually have disappeared. Repeated exposure can lead to the chronic condition, with progressive dyspnea and lung fibrosis. Sometimes a subacute form can also appear (16, 18). The common factor among the aforementioned diseases seems to be exposure to high levels of fungal diaspores (including those of actinomycetes).

The drying of sawn timber at sawmills today is mostly done artificially in special drying chambers, called kilns, of various types. The temperature in these kilns varies between 35 and 60°C, and the relative humidity is high. This environment creates favorable conditions for growth of thermophilic and thermotolerant fungi. The predominant organisms are Aspergillus fumigatus (Fres.), Paecilomyces variotii (Bain.), and Rhizopus rhizopodiiformis (Cohn) (15). After kiln drying, the timber is transferred to the trimming department of the sawmill, where it is trimmed to desired length and sorted for quality. When the incoming timber is contaminated with fungi, large amounts of diaspores can be introduced into the air by vigorous handling of the timber along the production line. Inhalation of large quantities of these fungal diaspores can induce pulmonary diseases among the sorters and the wood trimmers (1).

Tremorgenic mycotoxins are a group of substances reported to induce sustained or intermittent tremors in treated mice, rats, chickens, and sheep (6, 9, 17). The clinical symptoms of this neurological syndrome involve tremor, hypersensitivity to sound, tetanic spasms, incoordination (ataxia), and sometimes death, depending upon dose (9, 17). The exposure route for these mycotoxins is usually through moldy or contaminated fodder or other feedstuffs (8, 10, 17), but aflatoxin inhalation has been reported as a problem for workers handling dusty contaminated corn (4, 5) and peanuts (11, 14, 25), which in one case led to lethal alveolar cell carcinoma (11). Emanuel et al. (12) proposed that 10 patients who were exposed to large amounts of fungal diaspores during handling of silage developed acute toxic pulmonary reactions due to inhaled fungal toxins. Drugs causing sustained trembling are very rare. In one investigation (13), less than 10 of 10,000 screened compounds were found to have these properties, yet approximately 20 fungal metabolites are known to induce tremors in animals (7). Besides Claviceps paspali, tremorgenic mycotoxins are produced only by various Penicillium and Aspergillus spp. (2). A. fumigatus is known to produce at least five different tremorgenic toxins (fumitremorgens A, B, and C, verruculogen, and TR-2) (7).

The symptoms of the acute form of wood-trimmers’ disease and the common occurrence of A. fumigatus in the air of trimming departments prompted an investigation of the ability of these A. fumigatus strains to produce tremorgenic mycotoxins. This paper describes an investigation of eight strains of A. fumigatus from five different sawmills in Sweden.

MATERIALS AND METHODS

Tremorgenic mycotoxin standards. Verruculogen and fumitremorgens B and C were kindly provided by R. J. Cole.

Origin and isolation of fungi. The fungi were sampled from timber inside the kilns, from the walls and the floors of the kilns, and from the indoor air. The fungi were isolated on either 9-cm petri dishes (sedimentation) or “impress plates” (6-cm petri dishes with a convex surface that was pressed against the surface to be examined) containing MEAM.
medium (malt extract, 25 g; agar, 15 g; malic acid, 5 g; water, 1 liter). The plates were incubated at 40°C for 1 to 4 days, and then colonies were transferred to separate plates until pure cultures were obtained. The A. fumigatus strains were identified with the criteria of Raper and Fennell (22); of the eight strains used, five (A to E) were fresh isolates, and three (F to H) were taken from the culture collection at the Department of Forest Products, The Swedish University of Agricultural Sciences, Uppsala. The three latter isolates had been stored on 2.5% malt extract agar (MEA; 2.5% malt extract, 1.5% agar) under sterile paraffin oil for 7 years. Before use in the experiments, they were cultured on fresh MEA medium. All strains were isolated from progressive kilns except strain G, which was from a compartment kiln. The coniferous wood materials being dried in the kilns were from Scots pine (Pinus sylvestris) and Norway spruce (Picea abies).

Media and incubation for toxin production. Cultivation was done in a liquid medium by the method of Yamazaki et al. (26), but with ammonium tartrate instead of ammonium succinate. The strains were inoculated into 1-liter conical flasks, each containing 250 ml of medium. In one series of flasks for strains A, E, and G (two flasks per strain), the medium was supplemented with 125 mg of L-tryptophan per liter before autoclaving. In a second series, sterile filtered l-tryptophan, 250 mg/liter, was added to the medium after this medium was autoclaved. In this series strain A was inoculated into four flasks, G was inoculated into two flasks, and B, C, D, F, and H were inoculated into one flask each. All flasks were incubated at 30°C for 3 weeks. Strain A was also cultured on four pieces of gamma-sterilized sapwood blocks (2 by 2 by 2 cm) of Scots pine. A spore suspension in sterile water was prepared from MEA medium cultures and transferred to the blocks, giving an initial water content of 50% (percentage of dry matter). Two wood blocks per 100-ml conical flask were incubated as described above. Two sterile wood blocks were used as controls.

Extraction of liquid cultures. To each culture flask, 175 ml of chloroform was added and mixed for 30 s in a Waring blender. After filtration through a paper filter, the two phases were separated in a separatory funnel. The chloroform phase was evaporated to dryness on a rotary evaporator (water bath temperature, 45°C) and dissolved in 5 ml of ethyl acetate. The ethyl acetate was washed three times with aqueous HCl (pH 2) (centrifugation between each step), after which the ethyl acetate phase was removed from the aqueous phase, and evaporated to dryness under N2 on a water bath (45°C). This method was adopted from Cole et al. (8).

Extraction of wood cultures. The wood cultures were shaken for 30 min together with 25 ml of deionized water per wood block. Chloroform (25 ml per wood block) was added, and the mixture was shaken for another 30 min. The phases were separated in a separatory funnel, and the chloroform phase was evaporated to dryness, dissolved in 5 ml of ethyl acetate, and processed as described above.

Chemical analysis. The ethyl acetate was evaporated. The residue from an ethyl acetate extract was dissolved in 1 ml of toluene and passed through a silica gel column (Kieselgel 60; Merck) (35/70 mesh; internal diameter, 30 by 5 mm) which was then washed with 5 ml of toluene. The tremorgen was eluted with 5 ml of toluene-ethyl acetate-formic acid (5:4:1, vol/vol/vol). The solvent was evaporated, and the residue was dissolved in 1 ml of the mobile phase to be used in high-performance liquid chromatography. Some ethyl acetate extracts were divided into two parts, and verruculogen (25 µg) was added to one part as internal standard. Aliquots of the tremorgen samples (20 µl each) were run on a LiChrosorb RP column (pore size, 10 µm; internal diameter, 250 by 3.3 mm) with methanol-water (72:28, vol/vol) as mobile phase. A model 2150 pump and a model 2140 rapid spectral detector were used (LKB, Bromma, Sweden). With a flow of 0.5 ml/min, retention times for fumitremorgen C, verruculogen, and fumitremorgen B were 5, 11, and 18 min, respectively.

TABLE 1. Mycotoxins found in and tremorgenic reactions caused by extracts from A. fumigatus cultures isolated at sawmills

<table>
<thead>
<tr>
<th>Extract*</th>
<th>Sawmill</th>
<th>Tremorgenic reaction</th>
<th>Mycotoxin(s) found</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>a</td>
<td>Very strong</td>
<td>Verruculogen, fumitremorgen C</td>
</tr>
<tr>
<td>B</td>
<td>a</td>
<td>None</td>
<td>Verruculogen</td>
</tr>
<tr>
<td>C</td>
<td>a</td>
<td>Mild</td>
<td>Verruculogen</td>
</tr>
<tr>
<td>D</td>
<td>b</td>
<td>Mild</td>
<td>Fumitremorgen C</td>
</tr>
<tr>
<td>E</td>
<td>b</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>c</td>
<td>Mild</td>
<td>Fumitremorgen C</td>
</tr>
<tr>
<td>G</td>
<td>d</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>e</td>
<td>Mild</td>
<td>Fumitremorgen C</td>
</tr>
<tr>
<td>I</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oil 1</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oil 2</td>
<td>None</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Extracts were administered to 100-g rats orally in 1.5 ml of peanut oil; three animals were used in each group. Extracts A to H were from A. fumigatus on liquid media; extract AW was from wood culture of A. fumigatus A; extract I was from sterile liquid media; extract J was from sterile wood. Oil 1 was a control with peanut oil; oil 2 was a control with peanut oil plus added chloroform aerated with bubbles of nitrogen.

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The dominant fungal flora isolated from wood at the sawmills, which dry the timber in kilns, were A. fumigatus, F. variotii, and R. rhizopodiiformis. No quantitative measurements were done. The production of tremorgenic mycotoxins was tested only with A. fumigatus strains. Table 1 gives the results from animal tests and chromatography of the extracts. Extracts from strain A produced very strong tremorgenic effects, whereas extracts from strains C, D, F, and H produced mild tremorgenic effects. Strain A was also cultivated on wood, and the extract obtained, AW, had as strong an effect as that from liquid medium. The first toxic signs of A and AW extracts were noticed between 45 and 60 min after administration. The animals were sluggish, and a mild tremor could be provoked by acoustic (clapping of
hands) or tactile stimulation. Over time, the intensity of the tremors that could be provoked increased. After several hours the tremors occurred spontaneously. By 24 h postinoculation the tremors disappeared. One rat from the AW group was found dead the day after treatment. Two days after treatment all of the other animals appeared to be healthy and fully recovered. The only tremorgenic symptoms in animals treated with extracts C, D, F, and H were recorded in 1975. The tremors disappeared by 24 h postinoculation. Very strong tremorgenic reactions were observed in animals treated with extracts C, D, F, and H. These toxins are known to be produced by Aspergillus fumigatus (7). Verruculogen and fumitremorgen C, were found in the extracts. These toxins are known to be produced by A. fumigatus (7). Verruculogen and fumitremorgen C were identified on the basis of retention times, absorbance spectra, and the internal standards used in the high-performance liquid chromatography system. No quantitative measurements were done. The correlation between tremorgenic reactions and occurrence of mycotoxins was good except in two cases. Extract AW, obtained from wood, produced very strong tremors, but none of this extract was left for chemical analysis. In another case, extract B contained detectable amounts of verruculogen, but obviously the level was below that required to produce tremors.

**DISCUSSION**

The metabolism of verruculogen in animals is not fully understood, but studies with rats and sheep have indicated that it is metabolized in the liver to more polar products (including the tremorgen TR-2), which are excreted into the bile (20, 21). The toxin affects the central nervous system; its possible mode of action is by interference with cerebrocortical nerve endings (synaptosomes), causing changes in the amino acid neurotransmitter release mechanisms (17, 19, 23). Verruculogen is the most potent tremorgenic mycotoxin known, being tremorgenic when given intravenously to sheep or pigs in the range of 5 to 15 μg/kg of body weight (20). The presence of large amounts of A. fumigatus diapores in indoor air at sawmills may lead to an exposure to tremorgenic mycotoxins by inhalation. Such an exposure to these mycotoxins has not been studied but merits further attention in order to reveal a possible role in acute alveolitis. The occurrence of the fungus A. fumigatus as such in the indoor air in sawmills is serious enough, but the capacity of the isolated strains to produce tremorgenic mycotoxins will give the problem an even higher priority. Also, other groups of workers, for example, farmers, might be exposed because A. fumigatus is known to contaminate cereals which have been treated improperly and which have gained heat during storage.

**ACKNOWLEDGMENTS**

We thank R. J. Cole, U.S. National Peanut Research Laboratory, for the tremorgenic mycotoxin standards and Antun Fajdetic for technical help.

This work was partly supported by grants from the Swedish Council for Forestry and Agricultural Research and the Swedish Council for Building Research.

**LITERATURE CITED**


