Detection of Aflatoxins in Autopsied Materials from a Patient Infected with Aspergillus flavus

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Abstract

We isolated two strains of Aspergillus flavus from a lung lesion and a skin lesion at autopsy from a patient with acute myelogenous leukemia complicated with fungal infection. An attempt was made to detect aflatoxins in culture filtrates of those isolates and the tissue extract of the lung lesion through the techniques of thin-layer chromatography (TLC), densitometry and high-performance liquid chromatography (HPLC). Aflatoxins B₁, B₂ and M₁ were demonstrated in all of these materials qualitatively and quantitatively. The concentrations of aflatoxins in the cultures of the isolates and in the lung lesion extract determined by HPLC were aflatoxin B₁: 11.715 µg/ml (lung isolate), 21.383 µg/ml (skin isolate), 0.635 µg/g (lung extract), aflatoxin B₂: 0.341 µg/ml (lung isolate), 0.577 µg/ml (skin isolate), 0.0273 µg/g (lung extract) and aflatoxin M₁: 0.277 µg/ml (lung isolate), 0.491 µg/ml (skin isolate), 0.0525 µg/g (lung extract), respectively. B₁, known as the most toxic among the aflatoxin group, showed the highest concentration through these experiments.

This case may be considered as the first to detect aflatoxins in autopsied materials associated with A. flavus infection.

Key words: aflatoxin, Aspergillus flavus, aspergillosis, acute myelogenous leukemia, lung lesion

Materials and Methods

The patient was a 41-year-old man with neutropenia following induction therapy for acute myelogenous leukemia; his elevated body temperature and chest X-rays showing multiple nodular shadows with cavity formation suggested an Aspergillus infection. Attempts to lower the fever and improve the chest X-ray images by antifungal chemotherapy and subcutaneous recombinant G-CSF injection followed by bone marrow transplantation (BMT). The clinical data in this case will be reported elsewhere. This process prompted us to investigate the possibility of production of aflatoxins by infected A. flavus in tissues and also the possible influence of the etiological agents and their metabolites in establishing systemic infection in the patient.

This paper describes the productivity of aflatoxins in culture filtrates of the isolates and the lung tissue extracts using TLC, densitometry and HPLC.
by *Aspergillus* were observed in both lungs, with infarction of the right upper lobe. Such lesions were also seen in the brain, myocardium, epicardium, kidneys, thyroid gland and spleen. Multiple whitish subcapsular nodules were detected in the subcutaneous tissues.

No fungi had been isolated while the patient was alive, but the species *A. flavus* strains were isolated from lung and skin lesions at autopsy. Routine identification of the isolates was made, and they proved identical both morphologically and biologically.

These isolates were inoculated into YES broth containing 2% yeast extract and 15% sucrose, and incubated for eight days at 30°C in the dark as stationary cultures. To sterilize the cultures obtained, half was filtered through a filter (Millex GS, 0.22 μm, Millipore), and the other half was autoclaved at 121°C for 20 min. These culture filtrates were inoculated into YES broth containing 2% yeast extract and 15% sucrose, and incubated for eight days at 30°C in the dark as stationary cultures. To sterilize the cultures obtained, half was filtered through a filter (Millex GS, 0.22 μm, Millipore), and the other half was autoclaved at 121°C for 20 min. These culture filtrates were inoculated into YES broth containing 2% yeast extract and 15% sucrose, and incubated for eight days at 30°C in the dark as stationary cultures.

Lesions of the lungs, liver, kidneys, spleen, heart, brain and skin obtained at autopsy were preserved in 10% formol saline. Each tissue was washed with water thoroughly, cut into small pieces, and homogenized in distilled water at maximum speed for 20 sec several times using a Physcotron NS-60 (NITI-ON). The homogenized tissues were treated with hexane to remove lipid ingredients, followed by 20% basic lead acetate to improve the extraction efficiency. Extraction was done with chloroform. Each chloroform layer obtained was dried with ab. Na2SO4, evaporated to dryness, and dissolved in a benzene-acetonitrile (9: 1) solution. These sample solutions were spotted on a TLC plate (0.25 mm silica gel 60 thin-layer plate, Merck 1.05721) and developed with chloroform, acetone and hexane (85: 15: 20). After TLC, the plate was examined under high intensity ultraviolet light (365nm). The entities of aflatoxins therein were determined qualitatively by visual comparison with aflatoxin B1, B2, G1, G2 and M1 standards (Sigma A-6636, A-9887, A-0138, A-0263, A-6428). Quantitative analysis was performed by densitometry (Master scan; Interpretive densitometer) [Integration time; long, Light source; UV, Intensity gain; 8, Down sample; 4, F-stop; 2.8] The same samples were pre-treated for HPLC assay as follows: 1ml of chloroform layer was evaporated, the residue was resolved with a mixture of 100 μl of water and 100 μl of trifluoroacetic acid, heated at 70°C for 20 min, and after cooling, 0.3 ml of mobile phase was added. This solvent thus obtained was injected into the HPLC apparatus. [Pump; PU-980 (JASCO), Column; Nova-Pak Phenyl (5 μm, 3.9 mm×150 mm; Waters), Mobile phase; water : acetonitrile (85 : 15), Flow rate; 1.0 ml/min, Fluorescence detector (FP-920S, JASCO), Ex: 365 nm, Em: 440 nm]

### Results

Data on TLC, densitometry, and HPLC analysis are summarized in Table 1. Under ultraviolet light, the TLC experiment showed several spots suggesting blue fluorescent compounds in the culture filtrates and the extract of lung tissue. Compared with aflatoxin standards, they were identified as aflatoxin B1, B2 and M1 (Fig. 1) with a Rf value 0.73 (B1), 0.69 (B2) and 0.23 (M1), respectively. At the position of aflatoxin G1, a spot showing fluorescent compound was recognized, but the tone of color was different from G1. These results indicate that aflatoxin G1 and G2 were not present in any of the samples. On the TLC spots, RF values were found to be equal in the filtered culture filtrate and autoclaved culture filtrate, showing that those detected aflatoxins were heat-stable. It was now clear that

<table>
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<tr>
<th>Sample</th>
<th>TLC (Rf value)</th>
<th>Densitometry (μg/ml)</th>
<th>HPLC (μg/ml)</th>
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<tr>
<td>Culture filtrate</td>
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<tr>
<td><em>A. flavus</em> strain isolated from lung</td>
<td>0.736 0.696 0.236</td>
<td>13.66 1.54 1.91</td>
<td>11.715 0.341 0.277</td>
</tr>
<tr>
<td><em>A. flavus</em> strain isolated from skin</td>
<td>0.736 0.696 0.236</td>
<td>12.48 1.36 1.62</td>
<td>21.383 0.577 0.491</td>
</tr>
<tr>
<td>Tissue extract</td>
<td></td>
<td></td>
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<tr>
<td>lung</td>
<td>0.730 0.689 0.236</td>
<td>0.78* 0.11* 0.13*</td>
<td>0.635* 0.0273* 0.0525*</td>
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<tr>
<td>spleen</td>
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<td>liver</td>
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<td>brain</td>
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U: undemonstrable *: μg/g(dried weight)
Aflatoxin B1, B2 and M1 were contained in both the culture filtrates and the lung lesion extract. Aflatoxins were not detected from other organs. The concentrations of aflatoxin B1 showed similar values by densitometry and HPLC, but in B2 and M1 the differences were large. HPLC was more sensitive than densitometry (limits of detection were 1 ng/ml; HPLC and 10 ng/ml; densitometry), so we adopted the HPLC data (Fig. 2). The culture filtrates showed almost the same levels of aflatoxin contents in the two isolates. Among these three derivatives, the concentration of aflatoxin B1 was obviously the highest.

Discussion

We isolated *A. flavus* from the lung and skin lesions of a compromised patient complicated with systemic aspergillosis following antileukemic chemotherapy and BMT for acute myelogenous leukemia; the host died despite aggressive antifungal chemotherapy. We then attempted to demonstrate the production of aflatoxins in the infected tissues as well as from the causative fungus itself.

TLC is a simple and rapid method for mycotoxin analysis. With the advances in the chemistry of adsorption materials for column packing and improvements in instrumentation and enhancement of detector efficiency, HPLC has proved to be one of the most popular methods to detect a minute amount of mycotoxin. We therefore applied these methods to our study.

A number of clinical reports on acute aflatoxicosis and its relation to chronic hepatic injury and hepatocellular carcinoma due to aflatoxin contaminated foods have appeared. Aflatoxins are also known to affect and suppress the immune responses of experimental animals. However, no previous report has been made to our knowledge of the detection of aflatoxins from the lesions of a clinical case complicated with *A. flavus* infection.

Our patient had been suspected of having pulmonary *Aspergillus* infection from the first episode of infection to the end of his life, but clinically clear diagnostic evidence was not obtained until his death. The occurrence of systemic *Aspergillus* infection was proved at autopsy, and the causative fungus, *A. flavus* strains were isolated from the infected sites. It is of particular interest that the strains produced aflatoxins B1, B2 and M1 in the lung lesion.

In a preliminary experimental study, a comparison was made of incubation temperature of 30°C, which is generally an optimal temperature for fungal growth, and 37°C as the average
human body temperature to learn which caused greater toxin productivity in infected tissues. The culture filtrates of the isolates incubated at 37°C showed a lesser amount of aflatoxins than at 30°C. This can be attributed to slightly worse fungal growth and toxin production at 37°C. For the assay of aflatoxin productivity, the incubation at 30°C was reported to be the optimum condition19,20).

In our experiment, although the lung tissue extract contained a smaller amount of aflatoxins than the culture filtrates, the finding that even very small amounts of the toxins were present in the affected lung lesions but that none were demonstrated in other organs including liver are meaningful in our knowledge of the establishment and course of pulmonary aspergillosis. In other words, it may be speculated that A. flavus vigorously invades the lung tissues, and produces aflatoxins which damage these tissues and affect the immune system of the patient.

Pre-treatment of HPLC might be unsuitable for the detection of aflatoxins in tissue extract, because this extract might contain many components, thereby threatening to interfere with the detection of small amounts of aflatoxins during the extraction procedure28). A tailing spot was seen on the TLC plate and many peaks were detected by HPLC (figures cut out). We believe that one of the most important steps in aflatoxin analysis is the sample preparation.

Wei and Jong29) demonstrated that 40% of A. flavus strains produced aflatoxins. The possible relationship between the toxicity and pathogenicity of aflatoxin-producing A. flavus strain should be investigated in detail by animal models.

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References.


