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Isolation of Satratoxins from the Bedding Straw of a Sheep Flock with Fatal Stachybotryotoxicosis

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During a period of several weeks, more than 100 sheep died at a Hungarian farm. The animals exhibited fleece loosing, and hemorrhaging was the most important autopsy finding. *Pasteurella haemolytica* was cultured from various organs. The bedding straw was abundantly covered with *Stachybotrys atra*, and removal of the straw stopped the disease. Methanol extraction of the bedding straw followed by solvent partitioning, column chromatography, preparative thin-layer chromatography, and high-pressure liquid chromatography led to the isolation of satratoxins G and H, which were characterized by thin-layer chromatography, high-pressure liquid chromatography, and mass spectroscopy. This is the first isolation and characterization of toxins from a field sample of material responsible for an outbreak of stachybotryotoxicosis.

The trichothecene mycotoxins (2, 3, 6, 13, 22) pose serious risks to human and animal health around the world. Although the simple trichothecenes (e.g., T-2 toxin, diacetoxyscirpenol, and deoxynivalenol [2, 3, 6, 13, 22]) appear to constitute a more common and widespread hazard, the macrocyclic trichothecenes (e.g., roridins, verrucarins, and satratoxins [20; B. B. Jarvis and E. P. Mazzola, Acc. Chem. Res., in press]) have also been implicated in a number of cases (22).

Of particular interest to us is stachybotryotoxicosis, a disease caused by the toxins associated with the mold *Stachybotrys atra*. This organism has been associated with both human and animal health problems (1, 7, 18). In particular, infestations by the fungus give rise to serious economic losses in sheep (5), cattle (4), horse (7), and swine (18) breeding in eastern Europe. There have also been reports of similar animal toxicoses in France (12), Finland (23), South Africa (15), and India (14). Animals such as buffalo (17), European elk (23), dappled fallow deer (12), bison, and hippopotami (21) have also fallen prey to stachybotryotoxicosis. However, in all of these cases, diagnosis was based only on clinical signs, autopsy findings, isolation of *S. atra*, and demonstration of the toxicity of the straw or the culture of *S. atra* isolated from the straw. What have been missing are the isolation and characterization of toxic chemicals from the feed or bedding responsible for the toxicoses.

Several macrocyclic trichothecenes have been isolated from *S. atra* cultures in the United States (8, 9), Hungary (10), and Finland (11). The principal macrocyclic trichothecenes produced appear to be the satratoxins G and H (Fig. 1) (8, 9), which suggests that these agents are responsible for the toxicoses observed in the field. A method has been reported for the analysis of satratoxins from *S. atra*-contaminated grains (16); however, in the field, it is commonly straw that serves as the substrate for *S. atra*, since on grains the organism is usually overgrown by other fungi. The ability of *S. atra* to decompose cellulose enables it to be highly successful in competition with other fungi for use of straw as a substrate. Thus, of the material in contact with animals, only straw can be abundantly covered with *S. atra*.

Herein, we report the detection of satratoxins G and H in a sample of straw shown to be responsible for a serious outbreak of stachybotryotoxicosis in sheep. From the end of January through February 1982, over 100 sheep of a flock of 1,200 ewes on an eastern Hungarian farm died. Because of a lack of adequate winter feeding, these animals often consumed the bedding straw, which was visibly contaminated with black colonies of *S. atra*. The most characteristic clinical signs of illness were weakness and bloody nasal discharge. In some cases, diarrhea and fleece loosing were observed; some animals became almost nude. Treatment with antibiotics appeared to have no effect on the course of the disease.

MATERIALS AND METHODS

Autopsy findings. All but eight corpses were dissected by the local veterinary surgeon, and the remaining eight were transferred to Veterinary Institute, Debrecen, Hungary, for a more detailed examination. The autopsy findings showed the presence of extended hemorrhaging in the subcutis, on the mucosal surfaces, and under the serosal membranes. Frequently, the rumen contents consisted exclusively of bedding straw. In the Veterinary Institute, *Pasteurella haemolytica* was isolated from the spleens and lungs of four animals.

Toxin isolation and characterization. Unground straw (100 g) was extracted with methanol. After the methanol was removed, the resulting residue was partitioned between 100 ml of petroleum ether and 100 ml of water. The water layer was extracted with 100 ml of methylene chloride. The residue from the organic layer was placed on a column containing 15 g of silica gel packed in methylene chloride. Elution with ethyl acetate gave a residue which was subjected to preparative thin-layer chromatography (silica gel $60F_{254}$ plates; 0.5 mm; E. Merck AG, Darmstadt, Germany; developed with 5% methanol in methylene chloride). The band which was identical in R_f to those of satratoxins G and H was collected and extracted with acetone. Comparison with satratoxin standards (8, 10) by high-performance thin-layer chromatography (Silica Gel-60 F_{254} plates; developed with 20% acetone in methylene chloride; visualized with UV light followed by a visualization after use of 4-*p*-nitrobenzyl pyridine spray reagent [19]) indicated the presence of satratoxins G and H in the preparative thin-layer chromatography bands.

The satratoxins obtained from preparative thin-layer chromatography were subjected to high-pressure liquid chromatography (Polygosil 60-D 5 C18 reversed-phase column; methanol-water [2:8; 8:2]; Waters Associates apparatus), and the retention times were shown to be identical to those of satratoxin G and H standards (Fig. 2).

Mass spectral analysis (AGI MS 92 [Micromass Mass Spectrometric Co.] mass spectrometer; direct probe) of the material collected from the high-pressure liquid chromatography column established the presence of satratoxins G and H (Fig. 3) in the samples.

RESULTS AND DISCUSSION

The death of the sheep was presumed to be due to stachybotryotoxicosis for the following reasons. The disease arose suddenly, and when the moldy straw used as bedding was removed, the disease soon ceased. The animals consumed the moldy straw, as evidenced by the finding of the material during autopsy. This bedding straw was heavily infected with *S. atra*. The clinical signs and autopsy findings, especially the fleece loosing and intensive hemorrhaging, were similar to those seen in experimental stachybotryotoxicosis in sheep (5, 15). *P. haemolytica* was isolated from several animals, and pasteurellosis is a characteristic complication of stachybotryotoxicosis in sheep (5, 15). As in other cases of stachybotryotoxicosis (5, 15), antibiotic treatment was ineffective, indicating that bacterial infection was not the main cause of the disease.

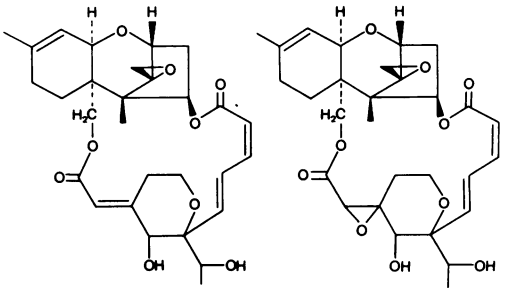

FIG. 1. Chemical structures of satratoxins G and H.

However, an adequate diagnosis of stachybotryotoxicosis should be based on or accompanied by the isolation and identification of the suspected toxins. Toxin production by *S. atra* under laboratory conditions does not demonstrate toxin production under field conditions, i.e., the cause of animal toxicoses.

We have clearly demonstrated for the first time that animal-ingested *S. atra*-infested straw contained satratoxins G and H, the presence of which was firmly established by thin-layer chromatography, high-pressure liquid chromatography, and mass spectroscopy (Fig. 2 and 3).

The isolation of two such highly toxic compounds from straw used for the bedding of the afflicted sheep suggests that the satratoxins were partly or mainly responsible for the disease, including the damage of the immune system which presumably led to the complication of pasteurellosis.

What has yet to be established is the precise role played by the macrocyclic trichothecenes in this type of toxicosis. The macrocyclic tricho-

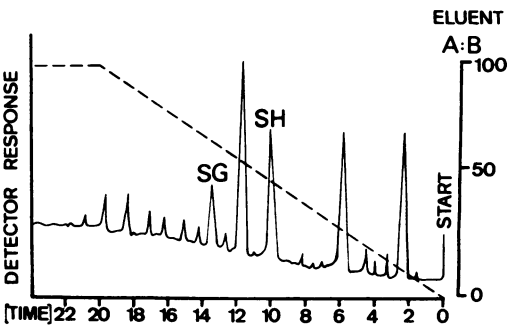

FIG. 2. High-pressure liquid chromatogram of the straw sample. Eluent A, Methanol-water, 8:2; eluent B, methanol-water, 2:8. SG, Satratoxin G; SH, satratoxin H.

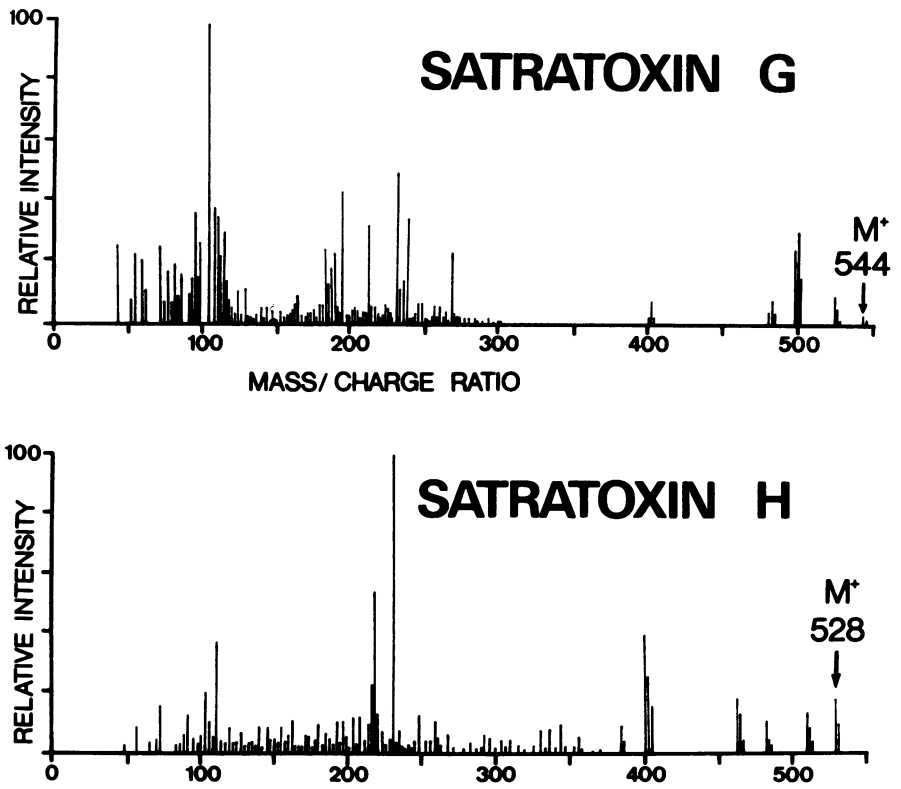

FIG. 3. Mass spectra of satratoxins G and H isolated from the straw sample.

thecenes are about an order of magnitude more toxic than the simple trichothecenes (e.g., the macrocyclic trichothecene verrucarin J, secondary metabolite of *Stachybotrys* and *Myrothecium* spp., has a 50% lethal dose value of 0.5 to 0.75 mg/kg for mice when injected intraperitoneally [2]). However, it remains to be demonstrated that satratoxins are present either individually or collectively in amounts sufficient to explain all of the effects noted in stachybotryotoxicosis. Such an evaluation must await the acquisition of these toxins in quantities sufficient for toxicological studies.

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