Bioconcentration and Biotransfer of Aflatoxin

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When certain molds of the Aspergillus flavus-oryzae species aggregate (1) contaminate food materials they excrete into the substrate several toxic and carcinogenic secondary metabolites known collectively as aflatoxins. Aflatoxin B_1, the most prevalent of the eight naturally occurring forms, is also the most toxic. When ingested in doses of from 0.5 to 10 mg/kg body weight, aflatoxin B_1 is lethal to many different animals. Perhaps more significant than the toxic effects are the carcinogenic results of low level ingestion of aflatoxin B_1; parts per million (ppm) or parts per billion (ppb) levels in feed results in tumor induction in several animal species (2). Prolonged exposure is not required; rats fed 0.4 mg aflatoxin B_1 over a 2 week period developed hepatomas 82 weeks later (3).

Since toxigenic A. flavus strains are relatively common contaminants of improperly harvested and stored grains and seeds, it is important to determine if other agricultural materials, particularly waste stockpiles, might harbor A. flavus and support its growth with subsequent aflatoxin formation. If this situation exists, the possibility of biotransfer and biomagnification of the toxin presents a potential environmental problem.

The present study reports the isolation of toxigenic strains of A. flavus from manure undergoing storage at a commercial feedlot. Laboratory investigation of a simulated food chain demonstrated that housefly maggots feeding on aflatoxin-contaminated manure incorporated aflatoxin into their biomass, thus rendering the toxin available to higher life forms. Rainbow trout demonstrated acute liver pathology characteristic of severe aflatoxicosis when fed maggots reared in manure substrates containing aflatoxin.

MATERIALS AND METHODS

The presence of A. flavus in stockpiled manure was determined by pooling samples from several regions of the manure stockpile at a commercial feedlot. Dilutions of 1:1000 were made with distilled water and 0.05 ml

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portions were added to pour plates of Czapek Solution Agar (Difco). After incubation at 28 C for 2 weeks, suspected A. flavus isolates were transferred to Sabouraud Dextrose Agar slants (Difco). The method of de Vogel (4) was employed to screen the A. flavus isolates for aflatoxin production.

To confirm toxigenicity, spores of the isolates obtained by the screening procedure were inoculated into Yeast Extract Sucrose medium (YES) (5) and incubated at 28 C. After 7 days, the cultures were extracted and analyzed for aflatoxin content (6). Identity of the extracted fluorescent metabolite corresponding in R_f value to an aflatoxin B_1 standard was confirmed by derivative formation (7), and toxicity was established by brine shrimp bioassay (8).

The most toxigenic isolate, designated A. flavus M-4, was tested for aflatoxin formation during growth on manure substrates. Fresh manure (collected from penned cattle) and aged manure (collected 5 cm below the surface of the pens) were dried at 60 C for 3 days. Flasks containing 10 gm dried manure and 20 ml tap water were autoclaved 10 minutes at 121 C and inoculated with approximately 10^9 spores of A. flavus M-4. After 7 days incubation at 28 C, the cultures were analyzed for aflatoxin production by extracting overnight with chloroform. The chloroform was filtered through glass wool and anhydrous Na_2SO_4 and evaporated under vacuum. The residue was dissolved in an appropriate volume of chloroform and analyzed by thin layer chromotography (9). Confirmation of identity was obtained as described above.

Maggots (Musca domestica larvae) were reared from eggs in moistened commercial dog food pellets. Methods for maintenance, harvesting, and storage were otherwise as described by Palm and Webster (10).

To determine percent conversion of substrate into maggot biomass, approximately 100 mg fly eggs were placed in 50 gm dried manure moistened with 70 to 100 ml tap water. After 7 days at 21 C, the maggots were harvested and weighed. The residual manure was dried to constant weight at 60 C and weighed.

Maggots for the fish feeding experiments were reared on moistened dog food pellets until they were approximately 10 mg in weight. To obtain toxic maggots, crude aflatoxin, extracted from the YES culture medium after growth of A. flavus M-4, was added to the dog food substrate, mixed thoroughly, and dried. Tap water was added to moisten and maggots were reared, harvested, and stored as previously described. The toxic maggots contained 5 ppm aflatoxin B and 10 ppm aflatoxin G and provided a dosage of 0.3 mg crude aflatoxin/kg fish/day.

Trout feed test diet was prepared as follows: pelleted feed (New Age Pellets, 3/32 ", J.R. Clark Co.) was soaked in an ethanol solution of aflatoxin B_1, mixed thoroughly and dried. This toxic feed provided approximately 1.6 mg B_1/kg fish/day. For the feed control diet, pellets were soaked in ethanol and dried.

Ten month old rainbow trout (Salmo gairdneri) averaging 50 gm were maintained in plastic troughs, each containing running well water at 16 C. At the time the trout were acquired, they were being fed twice daily 0.5 gm/fish/feeding and this schedule was maintained throughout the experiment. Each experimental set consisted of 14 trout. All trout were starved for 48 hours prior to the 10 day feeding period to ensure complete ingestion of food. After 10 days feeding, the trout were killed and necropsied. The livers were examined grossly and fixed 48 hours in 10% buffered formalin. Livers were sectioned in 2 dimensions, processed in the C.S.U. pathology laboratory and stained with hematoxylin eosin.

Aflatoxin B_1 was purchased from Calbiochem, Los Angeles and dissolved in chloroform prior to use. Aflatoxin standards (B_1, B_2, G_1, G_2) were obtained through the courtesy of Southern Utilization Research and Development Division, United States Department of Agriculture, New Orleans. Precoated silica gel G plates of 0.25 mm thickness were purchased from Brinkmann Instruments, Westbury, N.Y. Chloroform, acetone, 95% ethanol and anhydrous Na_2SO_4 were reagent grade.

RESULTS

From 1 gm pooled stockpiled manure, 50 suspected A. flavus isolates were obtained. In YES medium, isolates M-8 and M-9 produced 0.2 mg aflatoxin B_1 and 0.25 mg G_1/ml; M-4 produced 0.25 mg B_1 and 0.3 mg G_1/ml. On fresh manure , M-4 produced 2 µg B_1/gm and on aged manure, 0.2 µg B_1/gm.

To study biotransfer of aflatoxin from manure, maggots were chosen as the initial consumer in a simulated food chain. Over a 7 day feeding period at room temperature, maggots converted approximately 40% of a manure substrate into their biomass; this was equivalent to 35 mg substrate converted per 25 mg maggot. Second instar maggots feeding on substrates containing 0.02 ppm aflatoxin effected, in 2 days, a 10-fold bioconcentration of the toxin. Toxic maggots prepared for the fish feeding experiments tolerated crude aflatoxin concentrations of 1000 ppm in the substrate although relative bioconcentration was much lower. The aflatoxin content of maggots was found to depend on (1) amount of toxin added to the substrate, (2) temperature of incubation, (3) age of maggots and (4) length of time they fed on toxic substrate.

All trout survived the feeding experiment and evidenced no overt symptoms of toxicity or change in feeding activity, swimming behavior, or coloration.

Livers of the trout which had fed on non-toxic pellets were red-brown in color with smooth margins. Gall bladders were normal in size and partially filled with straw-colored fluid. There was no evidence of visceral hemorrhage. Gill coloration indicated normal blood levels. Microscopic sections showed normal liver architecture. Parenchymal cells were uniform in size and staining characteristics. The muralia were two cells wide and the sinusoids were distinct. The cells lining the bile ducts were consistant in size and staining. The duct lumens were clear.

Livers from the trout which had fed on non-toxic maggots were red-brown and slightly lighter in color than the livers of the trout which had fed on non-toxic pellets. There was marked prominence of the gall bladders which were about twice normal size and filled with a yellow fluid. Other than this, gross morphology was identical to that of normal trout. The liver architecture was normal; the bile ducts were clear. Microscopic findings were normal and were essentially identical to those of the control group.

The livers of the trout which had fed on toxic pellets were tan with pale edges and occasional interspersed white areas. Several livers showed petechiae. Gall bladders were somewhat larger than normal and were filled with light yellow fluid. Several fish had slight hemorrhagic streaking of visceral fat. Gill coloration indicated moderate anemia. Liver sections showed extensive cell destruction with fatty vacuolation and degeneration of parenchymal cells. Severe disturbance of liver architecture was widespread with some hemorrhagic necrosis, generally adjacent to the central veins. Trabecular fibrosis was evident in the periportal zones of several liver sections. Parenchymal cell nuclei varied in size and staining characteristics. Some nuclei appeared pale and swollen with margination of nuclear contents; others were pycnotic. The nuclei of the cells lining the bile ducts were distorted with moderate variation in stainability. The bile duct lumens were moderately occluded. One of the livers sectioned showed moderate bile duct hyperplasia. Liver regeneration was not observed. The microscopic picture was characteristic of severe degeneration due to aflatoxin toxicity.

Trout fed with toxic maggots had light tan livers. Moderately large gall bladders were filled with straw-colored fluid. Liver margins, though smooth, showed petechiae and small hemorrhagic areas. There was occasional hemorrhagic streaking of visceral fat. Gill coloration indicated moderate anemia. Microscopic

characteristics were identical to those of trout fed with toxic pellets. Abundant parenchymal cell degeneration and fatty vacuolation were the principal findings. There was little evidence of bile duct proliferation. The cells lining the bile ducts however, occasionally showed moderately distorted, non-uniform nuclei. Liver regeneration was not observed. The microscopic picture was identical to that of severe aflatoxicosis.

DISCUSSION

Toxigenic strains of A. flavus can be recovered readily from stockpiled manure. Although it was established that aflatoxin production occurs in manure under laboratory conditions, it remains unknown whether this also occurs in nature. During this study it was observed that many species of flies were present in feedlot areas. Accumulations of large masses of manure are attractive to ovipositing females and Musca domestica are reported to be the most predominant of the 7 fly species found in feedlots (11). It may be assumed that ample opportunity exists for the biotransfer of aflatoxin from manure stockpiles into insect larvae.

Maggots were shown to convert manure substrate into their biomass with an efficiency of 71%. When aflatoxin content of the substrate was 0.01 ppm, the efficiency of biotransfer of the aflatoxin was also 71%. It was noted that as the toxicity of the substrate increased, the efficiency of bioconcentration decreased. This observation, together with other unpublished data, suggests the induction or activation of an aflatoxin-transforming enzyme system and studies on this phenomenon are in progress.

The experimental results of dosing trout with aflatoxin were consistent with the reports of several workers (12,13). A dose of 1.6 mg aflatoxin B_1/kg fish/day elicited a severe aflatoxicosis within 10 days. Although no fish died during the test, extensive internal damage was observed on necropsy. Crude aflatoxin (approximately 0.3 mg/kg fish/day) bioconcentrated by maggots and ingested by the fish, resulted in essentially identical gross internal pathology and liver histopathology. It is unlikely that maggots would accumulate 15 ppm aflatoxin under field conditions and equally unlikely that trout could gain access to such toxic maggots. However, it is possible that flies developing from toxic maggots could retain enough aflatoxin to transfer a carcinogenic dose to trout. Dietary levels of 0.4 ppb aflatoxin induce trout hepatoma in 15 months (14). Thus, 1 gm flies (approximately 100) would need to retain only 0.0004 µg B_1 to attain a level capable of inducing hepatoma in a trout.

The evidence presented in this study indicates that aflatoxins in stored feedlot wastes can undergo bioconcentration and biotransfer. As is the case with other environmental pollutants, aflatoxin is most poorly understood in the aspect regarding the effects of low-level, long term exposure. The effects of chronic exposure to aflatoxin may be complicated by synergistic actions with other environmental pollutants and carcinogens (14,15).

Contamination of the environment with aflatoxin is probably most prevalent in agricultural areas with favorable high temperature and humidity (15,16). Since the limit of detectability of aflatoxin in natural products is presently on the order of 5 to 10 ppb (17), the method of directly analyzing agricultural wastes to detect the toxin is impractical. An additional complication in evaluating the significance of aflatoxin in the environment is that the carcinogenic or histopathological effects of low level exposure may not appear until months or years following ingestion of the toxin.

The recovery of toxigenic A. flavus from stockpiled manure and its demonstrated ability to form toxin during growth on manure substrates gain significance if biotransfer and biomagnification occur in nature as they have been shown to do in a simulated food chain. If these phenomena occur naturally, even minute amounts of aflatoxin in agricultural wastes present serious potential environmental health problems in view of the marked carcinogenicity of aflatoxin.

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