Biotransformation of Aflatoxin B₁ and Its Conjugated Metabolites by Rat Gastrointestinal Microflorases

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Rat cecal microflora from high- and low-fiber-fed animals hydrolyzed aflatoxin conjugates to metabolites indistinguishable from aflatoxin B₁ and aflatoxin P₁, but aflatoxicol was not a transformation product.

The gastrointestinal (GI) microflora can perform many diverse metabolic reactions, including hydrolysis of conjugates, dehydroxylation, decarboxylation, dealkylation, aromatization, deamination, reduction, and esterification of xenobiotics (14, 16). These activities may result in reabsorption of the released metabolites, activation or deactivation of xenobiotics (15), production of metabolites not formed in tissues (18), and possible variation in drug metabolism in different species and individuals.

Hepatic metabolism of aflatoxin B₁ (AFB₁), a carcinogenic mycotoxin produced by the fungi Aspergillus flavus and Aspergillus parasiticus, has been extensively studied, yet little is known about its metabolism by the GI microflora. In one study (12), AFB₁ was found to be transformed by rat fecal flora into a blue fluorescent compound, possibly aflatoxicol (AFL). The present study was conducted to investigate the metabolism of AFB₁ and its water-soluble conjugates by rat GI microflora, specifically the stomach and cecal microfloras, obtained from animals fed a low- or high-fiber diet. Diets of high-fiber content may affect the metabolic activity of the GI microflora (6) and decrease the incidence of colon cancer (1, 12).

Nonradioactive AFB₁ was purchased from Calbiochem, La Jolla, Calif. Ring-labeled [14C]-AFB₁ was prepared from cultures of A. parasiticus ATCC 15517 supplemented with sodium [1.14C]acetate (9). AFB₁ was purified by repeated thin-layer chromatography (TLC) on silica gel (Brinkmann Instruments Inc., Westbury, N.Y.), followed by high-pressure liquid chromatography (8). Standard AFB₁ and its metabolites AFL, AFP₁, AFQ₁, AFM₁, and AFB₂₄ were prepared as previously described (7, 13).

Urinary aflatoxin conjugates were isolated from Swiss Webster male mice (40 g, Simonsen’s Laboratories, Inc., Gilroy, Calif.) injected intraperitoneally with AFB₁ at 10% of the 50% lethal dose (40 μg of [14C]AFB₁ dissolved in 0.25 ml of dimethyl sulfoxide; specific activity, 0.45 Ci/mol). Mice were chosen to produce aflatoxin conjugates because of their relatively high metabolic activity (3). The urine was collected (free of fecal contamination for 2 days in ice-cooled graduated cylinders) from 8 to 10 animals housed in metabolism cages (Delmar Scientific Glass Products, Division of Coleman Instruments, Maywood, Ill.) under subdued light and was stored at −20°C before analysis.

After removal of particulate matter and chloroform-extractable substances, the urinary aqueous phase was passed three times through an Amberlite XAD-4 resin column (Rohm and Haas, Philadelphia, Pa.). The retained conjugates were eluted with acetone and methanol, dried, dissolved in a 0.2 M sodium acetate buffer (pH 5.0), and washed again with chloroform. The aqueous phase was then filtered (Millipore) and stored at −20°C.

Anaerobic medium (cecal medium) used for incubations was prepared by the method of Hungate (10) under an atmosphere of 100% CO₂ and had the following composition (in grams per liter): KH₂PO₄, 0.45; K₂HPO₄, 0.45; NaCl, 0.9; CaCl₂, 0.02; MgCl₂·6H₂O, 0.02; MnCl₂·4H₂O, 0.01; CoCl₂·6H₂O, 0.01; (NH₄)₂SO₄, 0.9; NaHCO₃, 5.0; cysteine·HCl·H₂O, 0.3; 10% rat cecal-intestinal extract. NaHCO₃ and cysteine were sterilized (autoclaved) separately. The cecal-intestinal extract was prepared under 100% CO₂ by mixing 100 g of cecal-intestinal contents and 200 ml of anaerobic (CO₂) water for 30 min, followed by centrifugation at 10,000 x g for 20 min to remove particulate material, and then was sterilized by autoclaving.

Cecal and stomach microflorases used in incubations were obtained from two 4-month-old male Sprague-Dawley rats (180 to 220 g, Small Animal Colony, Department of Animal Science, University of California, Davis) fed either a low-fiber (regular laboratory chow) or a high-fiber (regular laboratory chow plus 15% bran) diet.
upon weaning. Animals were sacrificed with chloroform. The ceca and stomachs were removed, placed in ice-cooled petri dishes, and transferred to an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.). The cecal or stomach contents from the two animals were squeezed into a beaker, and about 1 g of the pooled and mixed cecal or stomach contents was added to anaerobic culture tubes containing 8 ml of cecal medium. The tubes were removed from the anaerobic chamber and centrifuged for 10 min at 3,000 × g, and the clear supernatants were used as inocula for incubations.

A 1-ml sterile disposable syringe with a 21-gauge needle flushed with oxygen-free CO₂ gas was used to introduce anaerobically 0.2 ml of the microflora preparations and the substrates (30 μg of AFB₁; in 0.1 ml of dimethyl sulfoxide or 2 ml of conjugate preparation) into cecal medium, and cultures were incubated at 37°C for 2 days. Two experiments were performed on AFB₁ or on conjugates as substrates. Each experiment consisted of triplicate experimental and control tubes. Substrate-free and microflora-free controls were used.

After incubation, the hydrolysates were extracted from the culture with chloroform, concentrated, and analyzed by TLC. For cochromatography, some extracts were spiked with authentic aflatoxin metabolites and were placed at room temperature overnight. For one-dimensional TLC analysis, the plates were developed in chloroform-acetone-isopropanol (85:15:2.5 [vol/vol/vol]); for two-dimensional TLC analysis, the plates were developed in the above solvent and then in toluene-ethyl acetate-formic acid (6:3:1 [vol/vol/vol]).

The metabolites also were confirmed by autoradiography of the TLC plates for 1 month with Kodak medical No-Screen X-ray film (Eastman Kodak, Rochester, N.Y.). For radioactivity measurement, the metabolite spots were scraped off the TLC plate and transferred to scintillation vials containing 0.1 ml of distilled water to facilitate the release of silica-bound aflatoxin. After adding 13 ml of Handifluor (New England Nuclear Corp., Boston, Mass.), the vials were counted in a Tri-Carb liquid scintillation spectrometer (model 2425, Packard Instrument Co.) by using [¹⁴C]toluene (New England Nuclear Corp.) as an internal standard.

The rat cecal microflora was able to hydrolyze aflatoxin conjugates. Two compounds, chromatographically indistinguishable from AFB₁ and AFP₁, were released from mouse urinary conjugates (Table 1). Cecal microflora from the animals fed the high-fiber diet released more AFB₁ and AFP₁ than did the flora from animals on regular laboratory chow. This suggests that both AFB₁ and AFP₁ conjugates were present in mouse urine and that the dietary fiber content might enhance the metabolic activity of the cecal microflora.

The high-fiber cecal microflora transformed AFB₁ into five blue-green fluorescent compounds either more polar or less polar than AFB₁ (Table 2). The low-fiber microflora transformed AFB₁ into only three of the above five compounds missing the metabolites with Rf at 0.58 and 0.78.

The stomach microflora from both the high- and low-fiber diets also transformed AFB₁ into the three compounds. The apparent lack of effect of fiber content on the metabolic activity of the stomach microflora might have been due to short retention of fiber in the stomach.

Except for AFB₁, none of the blue-green fluorescent compounds had Rf values similar to those of the standard metabolites. It has been reported that AFB₁ was transformed to AFB₁ by Dactylhum dendroides (4, 5) and Rhizopus spp. (2) and possibly by rat fecal flora (11). However, AFB₁ and AFB₁ conjugates were prepared from mouse urine.

Table 1. Hydrolysis of aflatoxin conjugates* by rat cecal microflora

<table>
<thead>
<tr>
<th>Diet</th>
<th>Metabolite identified (% of aflatoxin conjugate)²</th>
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<tbody>
<tr>
<td>High fiber</td>
<td>AFB₁ (0.8), AFP₁ (2.4)</td>
</tr>
<tr>
<td>Low fiber</td>
<td>AFB₁ (0.3), AFP₁ (0.7)</td>
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* Aflatoxin conjugates were prepared from mouse urine.

² Two-dimensional TLC analysis and radioactivity measurement.

Table 2. Biotransformation of AFB₁, by rat cecal and stomach microflora*²

<table>
<thead>
<tr>
<th>Diet</th>
<th>Aflatoxin metabolites (Rf value)²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cecal flora</td>
</tr>
<tr>
<td>High fiber</td>
<td>BG (0.58), BG (0.69), AFB₁ (0.69)</td>
</tr>
<tr>
<td></td>
<td>AFB₁ (0.73), BG (0.78)</td>
</tr>
<tr>
<td></td>
<td>BG (0.82), BG (0.86)</td>
</tr>
<tr>
<td>Low fiber</td>
<td>BG (0.69), AFB₁ (0.73)</td>
</tr>
<tr>
<td></td>
<td>AFB₁ (0.82), BG (0.86)</td>
</tr>
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</table>

*² The microfloras were cultivated in cecal medium. The recovery rate of aflatoxin was 85 to 96% as measured by spectrophotometry.

One-dimensional TLC analysis. The fluorescent colors and Rf values of AFB₁ and its metabolites were as follows: AFB₁, blue, 0.72; AFL, blue, 0.66; AFQ₁, green, 0.46; AFM₁, blue, 0.31; and AFB₉₉, blue, 0.28.

BG, Blue-green fluorescent spot.
developing the TLC plates in the two solvent systems (ethyl ether and benzene-95% ethanol [40:6, vol/vol]) that gave characteristic $R_f$ values for AFL confirmed that none of the biotransformation products was AFL.

Our results therefore indicate that (i) rat GI microflora was able to transform AFB$_1$, (ii) the pattern of transformation was completely different from that of the hepatic system, and (iii) cecal microflora was able to cleave water-soluble aflatoxin conjugates formed in the liver. Conceivably, the released AFB$_1$ metabolites could be reabsorbed by the animal, completing the enterohepatic circulation of the toxin to prolong exposure. Thus, high-fiber diets might render the animal more susceptible to AFB$_1$ toxicity.

It was not expected that AFB$_1$ was a major product of hydrolysis of aflatoxin conjugates because it did not contain a hydroxyl group. Yet the compound was indistinguishable from AFB$_1$ in at least four TLC systems, and it was recovered in all of the 10 hydrolysis experiments performed (17). Studies are underway to further identify this metabolite, to elucidate the mechanisms of its conjugation, and to compare the activities of GI microflora under in vitro and in vivo conditions.

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