Carcinogenicity of Dietary Aflatoxin M₁ in Male Fischer Rats Compared to Aflatoxin B₁

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ABSTRACT

Aflatoxin M₁ (AFM), an hydroxy metabolite of the potent carcinogenic mycotoxin aflatoxin B₁ (AFB) is frequently found in milk and other dairy products. Sufficient amounts of AFM were produced to study the carcinogenicity of this compound. AFM was fed to male Fischer rats starting at 7 weeks up to 21 months of age. Agar-based semisynthetic diets contained 0.0, 0.5, 5.0, and 50.0 μg/kg of AFM or 50 μg/kg of AFB. Hepatocellular carcinomas were detected in two of 37 rats and neoplastic nodules were found in six of 37 rats fed 50 μg/kg AFM between 19 and 21 months. No nodules or carcinomas were observed in the lower AFM dose groups. Nineteen of 20 rats fed a diet containing 50 μg/kg of AFB developed hepatocellular carcinomas by 19 months of age. Carcinogenic potency of the aflatoxins was reflected by morphometric quantitation of foci detected in hepatocytin and eosin stained sections. Three rats fed the diet containing 50 μg/kg AFM developed intestinal carcinomas. None were observed in other groups. Under the conditions of this experiment AFM was found to be a weak hepatic carcinogen compared to AFB and to possess intestinal carcinogenicity.

INTRODUCTION

The presence of a toxic factor in the milk of cows ingesting AFB was first demonstrated by Allcroft and Carnaghan (1, 2). The toxic compound was subsequently identified as AFM, a chromatographically distinct hydroxy metabolite of the potent carcinogenic AFB (3, 4). The frequent presence of AFM in milk and other dairy products destined for human consumption has raised widespread concern regarding the health risk associated with the consumption of these contaminated dairy products (5–8). AFM contamination of milk in the Southeast and Southwest of the USA was observed on several occasions in the late 1970s in which up to 50% of samples from some areas were found to be contaminated by AFM at levels less than 1 μg/kg (ppb) (9). In 1977, the U.S. Food and Drug Administration established an action level of 0.5 ppb of AFM in fluid milk (10).

The acute toxicity of AFM was found to be nearly equivalent to that of AFB in rats and ducklings (11, 12). AFM is also genotoxic and injurious to cultured rat hepatocytes (13–15). Studies of the carcinogenicity of natural AFM undertaken in the rainbow trout and limited studies with synthetic racemic AFM in rats have indicated that AFM is less potent than AFB (16–18). Because of limitations in the supply of natural AFM, the carcinogenic potency of this compound has not been previously assessed by a chronic feeding study in rats.

We have been able to produce sufficient quantities of AFM using rice cultures of the fungus Aspergillus flavus NRRL3251 to determine the carcinogenicity of this compound in the male Fischer rat.

MATERIALS AND METHODS

Specific-pathogen-free 28-day-old, weanling male Fischer 344 rats were supplied by Charles River Farms (Wilmington, MA). They were housed individually in clear plastic cages with wood shavings for bedding and maintained at approximately 21°C in a 12-h light and 12-h dark cycle. During the first week, they were fed Purina Rodent Chow No. 5002 and then switched to an agar-based semisynthetic diet which contained casein as the major source of protein, as described by Wogan and Newberne (19). At 7 weeks of age, the animals were randomized into six treatment groups of approximately equal mean weights. Sixty-two animals were assigned to each of three treatment groups receiving 0.5 (AFM 0.5), 5.0 (AFM 5.0), and 5.0 (AFM 50) ppb of AFM in their diet, and a fourth group of 63 animals which received the agar-based diet without AFM. Forty-two animals were assigned to a positive control group receiving 50 ppb of AFB. An additional 42 rats were fed Purina Rodent Chow No. 5002 (control chow) to assess whether the weight gain of the rats fed the semisynthetic agar diets was equivalent to that of the rats receiving control chow. The control chow was analyzed chromatographically several times for aflatoxin and none was detected. All animals were offered food and water ad libitum. Animal weights were recorded weekly for 6 months and then monthly for the duration of the experiment. Food consumption was measured for a 24-h period each week for the first 6 months and 1 day per month for the remainder of the experiment. The total amount of aflatoxin ingested was estimated on the basis of the food consumption data. Rats sacrificed at the end of the study that had been fed 50 ppb of AFM had ingested approximately 1 mg of AFM. Rats from the 5.0 ppb and 0.5 ppb groups ingested 0.1 mg and 0.01 mg, respectively. The AFB group fed 50 ppb had received a total 0.8 mg of AFB by the time they were sacrificed.

AFM and AFB were prepared from strain NRRL3251 of Aspergillus flavus grown on rice culture, using a technique modified from that of Stubblefield et al. (20, 21). Briefly, yeast extract (2%), ZnSO₄ (26 μg/g), and water (4%) were added to 50-g portions of long grain rice and shaken continuously in 500-ml conical flasks at 28°C in subdued lighting for 10 days to improve the yield (21). The aflatoxins produced were extracted with chloroform. Clean up and final purification were performed using normal-phase column chromatography followed by reversed-phase chromatography (22). The chromatographically pure AFM extracted from the fungal cultures was indistinguishable from the authentic compound prepared by the biotransformation of AFB by using rat hepatic microsomal enzymes (22). Further confirmation of the identity and purity of AFM was obtained by UV, mass, and NMR spectroscopy as well as optical rotation.

AFM or AFB was dissolved in reagent grade acetone, and this solution was mixed into the portion of the corn oil used to prepare the agar diet. The toxin-containing oil was added to the molten agar prior to incorporation of other ingredients into the diet. No special attention was directed to the removal of acetone from the diet. The final concentrations of aflatoxins in the diet were confirmed by analysis using the method of Eppley (23). Fresh diet was prepared weekly from the stock mixture and stored at 20°C and stored in the dark.

Animals were sacrificed at approximately 3, 6, 12, 18, and 22 months. At least three animals per group were necropsied at the first three time periods. Larger numbers of rats were killed at later time periods. The condition of animals fed AFB began to deteriorate around 18 months and all remaining animals were necropsied at that time. Those fed AFM began to lose weight and their general condition deteriorated later and all remaining animals were sacrificed between 18 and 22 months of age.

At necropsies performed during the first 6 months of the experiment,
blood was drawn for multichannel serum analysis (SMA-12) and complete blood count to evaluate overall health and to check for indications of hepatotoxicity. Complete gross pathological examinations were performed. Particular attention was given to liver, lung, kidney, and intestine. The number, size, site, and appearance of hepatic lesions were recorded. The incidence of hepatocellular carcinomas was analyzed by the Fischer-Irwin Exact Test. Sections of liver, lung, and kidney were routinely collected and fixed in 10% buffered neutral formalin. Any abnormal appearing tissues from other organs were also processed for microscopy. These tissues were processed routinely, embedded in paraffin, cut at 6 μm and stained with hematoxylin and eosin for light microscopy.

The presence of preneoplastic foci, neoplastic nodules, and hepatocellular carcinomas in sections from the right, left, and median liver lobes was evaluated by light microscopy. Histological typing of hepatic lesions was performed on the basis of previously published criteria (24).

Morphometry. Morphometric quantitation of foci was performed for three time periods: period 1, 84–88 weeks; period 2, 90–94 weeks; period 3, 96–100 weeks. At least two slices from each of the right, left, and median lobes were examined from each of the animals in all groups. These slices were processed for light microscopy as described above.

The surface area of each liver slice was determined by first projecting the images of the samples using a stereomicroscope and then tracing the outline of each sample. The surface area of the tracing was determined by a digitizing pad (HIPAD; Houston Instruments, Austin, TX) connected to a microcomputer (LSI 11-23, Digital Equipment Corp., Maynard, MA). Approximately 8–10 cm² were examined per animal. The area of individual foci was determined by a point-counting technique which employed a double-square lattice test grid which had 80 points (25). The liver samples were projected from a microscope onto a high-resolution video screen with a final magnification of 64×. These data were used to calculate three morphometric parameters: (a) the volume fraction of liver which contained foci (Fv%), (b) the number of foci per unit area of liver (Nf), (c) the average area of foci A. Each treatment group was compared to other groups from the same time period and at different time periods. Statistical evaluation of morphometric data was performed using group means by analysis of variance (P7D of BMDP program) (26).

RESULTS

Analysis of serum enzymes indicative of liver injury (AST, ALT, and LDH) from aflatoxin-dosed groups and agar diet control rats was performed on blood collected at necropsy. No differences between dose groups and controls were detected in the first 6 months of the experiment, indicating that the doses of aflatoxin employed did not produce measurable hepatic toxicity. Minor elevations of these enzymes occurred in the hepatic carcinoma-bearing rats fed 50 ppb of AFB at the 17-month sacrifice period. Comparison of the slopes of growth curves of commercial chow fed rats with rats fed the semisynthetic agar diet indicated that the agar diet was adequate to produce growth equal to that of the commercial chow fed rats. During the first 6 months the AFM 0.5 ppb group grew significantly more rapidly than the chow fed rats.

Morphology of Hepatic Lesions. Rats fed AFB were the earliest to develop histologically detectable foci, at 10 months of age (Table 1). By 16 months, eight of nine rats had neoplastic nodules and by 17 months, carcinomas were present in 19 of 20 rats. In both periods the frequency of hepatic carcinomas was significantly greater than that observed at 21 months in rats fed 50 ppb of AFM. Foci were most often composed of a single cell type, usually eosinophilic in both AFM and AFB exposed groups (Fig. 1). Neoplastic nodules were most often composed of varying proportions of basophilic, eosinophilic, and clear cells. Hepatocellular carcinomas were most often composed of a mixture of trabecular and glandular patterns.
CARCINOGENICITY OF AFLATOXIN M1

Fig. 1. Histological appearance of an eosinophilic focus. Cells which comprise the focus can be distinguished from adjacent parenchyma by their larger size and uniform staining of the cytoplasm. H & E, x 85.

Fig. 2. Hepatocellular carcinoma in the liver of a rat fed a diet containing 50 μg/kg of AFM for 21 months.

One anaplastic hepatocellular carcinoma occurred in a rat from the AFB group sacrificed at 17 months.

Rats fed 50 ppb of AFM first developed foci at 16 months. One rat developed a neoplastic nodule by 17 months. Two of 10 developed nodules by 19 months and four of 18 had nodules by 21 months. Two animals from this group developed hepatocellular carcinomas by 21 months. One rat developed a neoplastic nodule by 17 months. Two of 19 developed nodules by 19 months and four of 18 had nodules by 21 months. Two animals from this group developed hepatocellular carcinomas by 21 months of age (Figs. 2 and 3).

Rats fed 5.0 and 0.5 ppb AFM first developed foci at 19 months and none were observed at 18 months. In period III, the number of foci per unit area of liver (Nf) was elevated in the AFB 50 group relative to all other AFM groups and control agar rats in period III. The value for the AFB 50 group from period I was greater than that of the AFM 50 group from period III, which had an additional 4 months of exposure. In period III, the Nf of the AFM 50 group was greater than the AFM 5.0 and agar control group. A progressive increase in Nf in the AFM 50 group with time was observed, but this trend was not observed in other AFM groups.

The mean area of foci (A) from the AFB 50 group was greater than that of AFM groups and agar control rats from period I. In periods II and III, the average areas of foci from the AFM 50 group were equivalent to those from the AFB 50 group at period I and greater than all agar control groups and lower dose AFM groups.

Nonhepatic Neoplasms. Intestinal adenocarcinomas were found in three animals exposed to 50 ppb of AFM. Two neoplasms involved the small intestine; one occurred at 9 months, and the other at 18 months. The third occurred in the colon following 19 months of exposure. All three were characterized by a polypoid growth pattern projecting into the lumen of the bowel, and partially occluding it. Hemorrhage from the surface of the neoplasms stained the feces distal to the tumor a deep red brown. Neoplastic epithelial cells invaded the adjacent submucosa and muscularis, forming poorly differentiated acini (Fig. 4). There was no evidence of metastasis by any of these neoplasms. No intestinal neoplasms were found in the other AFM groups, the AFB group, or the control group.

Compound tooth germ neoplasms occurred in 19 rats. Groups affected, in order of frequency were, AFM 5.0 (10/62), control agar (7/63), AFM 50 (1/62), and AFM 0.5 (1/62). All neoplasms were unilateral and arose from the maxillary incisors. The first tumor was observed at 17 months of exposure and the majority were observed at the last sacrifice period (22 months). These tumors did not appear to be related to AFM or AFB exposure. They will be described in detail in a subsequent communication.

Pulmonary adenomas were diagnosed in four animals. Two occurred in the AFM 50 group following 20 and 21 months exposure and two occurred in agar-fed control rats. Both were found 20 months from the start of the experiment. All were spherical and approximately 0.5 cm in diameter. Histologically
CARCINOGENICITY OF AFLATOXIN M₁

Table 2 Morphometric parameters for preneoplastic hepatic foci from rats continuously exposed to dietary aflatoxin B₁, M₁, or control diet

<table>
<thead>
<tr>
<th>Dietary level (ppb)</th>
<th>AFM 50</th>
<th>AFM 5.0</th>
<th>AFM 0.5</th>
<th>Control agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (months)</td>
<td>17</td>
<td>19</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td>No. rats</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Vₙ (mm³/mm³)</td>
<td>8.717±</td>
<td>0.150</td>
<td>1.165</td>
<td>1.182±</td>
</tr>
<tr>
<td>Nₙ</td>
<td>±2.202</td>
<td>±0.150</td>
<td>±0.399</td>
<td>±0.330</td>
</tr>
<tr>
<td>Vₚ (mm³)</td>
<td>3.417±</td>
<td>0.033</td>
<td>0.425</td>
<td>0.727±</td>
</tr>
<tr>
<td>Nₚ</td>
<td>±0.399</td>
<td>±0.033</td>
<td>±0.144</td>
<td>±0.198</td>
</tr>
<tr>
<td>dₚ (mm)</td>
<td>24.267±</td>
<td>7.417</td>
<td>28.525</td>
<td>16.700</td>
</tr>
<tr>
<td>Aₜ (cm²)</td>
<td>±3.699</td>
<td>±7.417</td>
<td>±7.118</td>
<td>±2.298</td>
</tr>
</tbody>
</table>

* Values are group means ± standard error at the mean.
* Vₙ, volume fraction; Nₙ, no. of foci/area of liver; Aₚ, average area of focus; Aₚ, average area of liver examined.
* Vₚ, AFM 50 > AFM 50, AFM 5.0 AFM 0.5 and control agar at 17 mo (P < 0.05) and AFMₚₙ and control agar at 21 mo.
* Vₚ, AFM 50 > M 5.0, control agar at 21 mo (P < 0.01).
* Nₚ, AFM 50 > M 5.0, AFM 5.0, AFM 0.5 and control agar at 17 mo (P < 0.01) and AFM 50 and control agar at 21 mo (P < 0.01).
* Nₚ, AFM 50 > AFM 5.0 and control agar at 21 mo (P < 0.01).
* Aₚ, AFM 50 > AFM 50, AFM 5.0, AFM 0.5 and control agar at 17 mo (P < 0.05).

The results of this investigation indicate that AFM is a hepatic carcinogen, although it is considerably less potent than AFB. By 17 months of age, 19 of 20 rats fed 50 ppb of AFB had multiple hepatic carcinomas, while at the same time, none were observed in rats fed AFM at the same level. Following 4 months of additional exposure, two animals fed AFM developed single hepatocellular carcinomas. Based on a comparison of the tumor incidence in AFM-fed rats in this study with the results of Wogan et al. (30) in a similar chronic study of AFB, AFM is about 2-10% as potent a carcinogen as AFB. Both results of Wogan et al. (30) in a similar chronic study of AFB, and confirmed the estimate of relative carcinogenicity based on nodules and carcinomas. AFB produced greater Vₙ and Nₙ of foci than AFM and produced the lesions earlier than AFM. Similarly, neoplastic nodules and hepatocellular carcinoma developed in the AFM group earlier and in greater numbers. A dose-related response and a temporal effect can be seen in the groups exposed to AFM. Increased Vₙ and Nₙ were observed with longer exposure to AFM and the highest values for these parameters were associated with the highest dose of AFM. Virtually no lesions were seen in the agar-fed control diet groups. The foci produced by AFM fed at 5.0 ppb and 0.5 ppb were statistically indistinguishable from those of the control animals. This suggests that these doses may have been below the threshold level of carcinogenicity, although adequate numbers of animals were available for statistical analysis. These findings suggest that the current FDA action level of 0.5 ppb for AFM in milk provides adequate protection for adult consumers. Whether infant animals are significantly more susceptible to the carcinogenicity of AFM than adults is not clear at present, although greater susceptibility of neonates to the carcinogenicity of AFB has been suggested (32).

In this study, three rats from the AFM 50 group developed intestinal carcinomas, while no intestinal neoplasms were observed in any of the other groups. Low numbers of mucinous adenocarcinomas of the intestine (2/246) of rats have been reported following AFB administration (33). Spontaneous intestinal carcinomas are rare in Fischer rats (27-29). Since no intestinal neoplasms occurred in 17 months of the AFB-exposed rats, the presence of three intestinal carcinomas in the group of as many hepatic carcinomas as AFB (16, 31). A racemic mixture of synthetic AFM has been evaluated in rats which received a total of 1 mg in 40 doses (18). Not until 96 weeks of exposure was a single hepatocellular carcinoma observed in one of 29 male Fischer rats. In the present study two hepatocellular carcinomas were observed earlier (86 weeks) and neoplastic nodules or foci were found in a higher proportion of animals. The racemic form of AFM may have less potency than natural AFM, if only one isomer is biologically active (12). The duration of the carcinogenic potency of AFM may be less than that of AFB, producing about one-third of the tumor incidence in AFM-fed rats in this study with the results of Wogan et al. (30) in a similar chronic study of AFB, and confirmed the estimate of relative carcinogenicity based on nodules and carcinomas. AFB produced greater Vₙ and Nₙ of foci than AFM and produced the lesions earlier than AFM. Similarity, neoplastic nodules and hepatocellular carcinoma developed in the AFM group earlier and in greater numbers. A dose-related response and a temporal effect can be seen in the groups exposed to AFM. Increased Vₙ and Nₙ were observed with longer exposure to AFM and the highest values for these parameters were associated with the highest dose of AFM. Virtually no lesions were seen in the agar-fed control diet groups. The foci produced by AFM fed at 5.0 ppb and 0.5 ppb were statistically indistinguishable from those of the control animals. This suggests that these doses may have been below the threshold level of carcinogenicity, although inadequate numbers of animals were available for statistical analysis. These findings suggest that the current FDA action level of 0.5 ppb for AFM in milk provides adequate protection for adult consumers. Whether infant animals are significantly more susceptible to the carcinogenicity of AFM than adults is not clear at present, although greater susceptibility of neonates to the carcinogenicity of AFB has been suggested (32).
rats fed AFM at 50 ppb warrants further investigation. AFM is more polar than AFB and is therefore poorly absorbed from the digestive tract. Retention in the digestive tract may be associated with the higher incidence of intestinal carcinomas. AFM, like AFB, requires metabolic activation for its mutagenicity and carcinogenicity (34). While this usually occurs in the liver by the cytochrome P450 system it may occur in the intestine, by the prostaglandin H synthase system, as recently reported by Battista and Marnett (35).

In the United States, human exposure to AFM occurs by ingestion of milk and other dairy products. The average human ingestion of AFM based on contamination levels of milk has been estimated to be 0.11 ppb (36). This is below the levels which were noncarcinogenic in male Fischer rats in this study. Human susceptibility to AFB-induced hepatocarcinogenesis is believed to be consistently lower than that of Fisher rats. This is based on the low susceptibility of primates to aflatoxin-induced carcinogenesis and the similarity of AFB metabolism by postmitochondrial fractions of human and monkey liver compared to that of the Fischer rat (37–39). Since AFM is considerably less potent than AFB the risk of hepatocellular carcinoma induced by AFM appears to be low. However, more work is indicated to assess the risk of this compound to human infants, since neonatal animals have been reported to be more susceptible than adults to experimental hepatic carcinogenesis by AFB, the parent compound of AFM (32).

REFERENCES
Carcinogenicity of Dietary Aflatoxin M<sub>1</sub> in Male Fischer Rats Compared to Aflatoxin B<sub>1</sub>

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