Induction of Osteogenic Sarcomas and Tumors of the Hepatobiliary System in Nonhuman Primates with Aflatoxin B,

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ABSTRACT

The carcinogenicity of aflatoxin B, (AFB,) has been under evaluation in nonhuman primates for the past 13 years. A total of 47 Old World monkeys, chiefly rhesus and cynomolgus, have received AFB, i.p. (0.125 to 0.25 mg/kg) and/or p.o. (0.1 to 0.8 mg/kg) for 2 months or longer, and 12 are currently alive and without evidence of tumor. Thirteen of the 35 monkeys necropsied to date (37%) developed one or more malignant neoplasms, yielding an overall tumor incidence of 28%. Five of the neoplasms were primary liver tumors (2 hepatocellular carcinomas and 3 hemangioendothelial sarcomas), and 2 cases of osteogenic sarcoma were found. Other tumors diagnosed were 6 carcinomas of the gall bladder or bile duct, 3 tumors of the pancreas or its ducts, and one papillary Grade I cystadenoma of the urinary bladder. The tumors developed in animals receiving an average total AFB, dose of 709 mg (range, 99 to 1354 mg) for an average of 114 months (range, 47 to 147 months). Fifteen of the 22 necropsied monkeys (68%) without tumor showed histological evidence of liver damage, including toxic hepatitis, cirrhosis, and hyperplastic liver nodules. These animals had received an average total AFB, dose of 363 mg (range, 0.35 to 1368 mg) for an average of 55 months (range, 2 to 141 months). Our results indicate that AFB, is a potent hepatotoxin and carcinogen in nonhuman primates and further support the hypothesis that humans exposed to this substance may be at risk of developing cancer.

INTRODUCTION

The carcinogenic effects of AFB, are being evaluated in long-term studies using nonhuman primates. We reported previously (3, 4) that 3 of 42 monkeys (7%) necropsied after receiving treatment with AFB, for longer than 2 years developed malignant primary liver tumors. The present report is an update of that study and describes 14 malignant tumors developing in 10 additional monkeys receiving long-term treatment with AFB,.

MATERIALS AND METHODS

The monkey colony consists of 540 animals and is composed of rhesus (Macaca mulatta), cynomolgus (Macaca fascicularis), and African green (Cercopithecus aethiops) monkeys. Details of maintenance and management procedures and of the method used to rear neonates have been described elsewhere (1, 13). Briefly, monkeys are separated from their mothers a few hr postpartum and hand reared in a nursery. They receive Similac formula until the age of 6 months and are then maintained on a diet of Purina monkey chow supplemented by one-half an apple and a vitamin sandwich (13) each day.

The monkeys are housed individually, and various clinical, hematological, and biochemical parameters are monitored to evaluate their general health. Tuberculin skin testing is done bimonthly. Blood is collected weekly or biweekly from a femoral vein into heparinized tubes. Routine hematological examinations (hematocrit, RBC, WBC, platelet counts, hemoglobin levels, and differential counts), and other clinical tests including alkaline phosphatase, total bilirubin, serum glutamic-pyruvic transaminase, and serum glucose-oxaloacetic transaminase are performed on all treated and control monkeys. Serum is also screened for the presence of AFP by double diffusion in agar gel with adsorbed antiserum to AFP and control serum. Further quantification of AFP in positive specimens is accomplished using a double-antibody radioimmune assay (27). The lower limit of sensitivity for the standard curve is 5 ng/ml. Serum AFP levels in control monkeys average 15.5 ng/ml for rhesus and 13.0 ng/ml for cynomolgus.

AFB, (Calbiochem, Los Angeles, Calif., and Makor Chemicals Ltd., Jerusalem, Israel) is dissolved in a minimum of DMSO and administered according to a variety of schedules by i.p. injection, nasogastric intubation, or by inclusion on a vitamin vehicle-treated control monkeys receive weekly doses of DMSO (0.2 ml/kg) by intubation. Drug or vehicle treatment usually begins within 10 days after birth and continues indefinitely or until tumor is detected.

When clinical evidence of liver lesions or an elevation of serum AFP appears, a laparotomy is performed under pentothal hydrochloride or ketamine hydrochloride anesthesia, and all lobes of the liver are carefully inspected for gross pathological changes. When such changes are found, a wedge biopsy of the affected lobe or apparent tumor is performed. Monkeys that die or are sacrificed are carefully necropsied, and the following tissues and organs are excised and placed in buffered formalin: brain; pituitary; salivary gland; thyroid; parathyroid; thymus; tongue; cheek pouch; trachea; esophagus; lungs; heart; aorta; liver; gall bladder; spleen; kidney; adrenal; stomach; pancreas; duodenum; jejunum; ileum; large intestine; lymph node; urinary bladder; testes; prostate; seminal vesicle (or ovary and uterus); skin; long bone; bone marrow; and tumor (tissue or mass). Paraffin sections are prepared for microscopic evaluation and stained with hematoxylin and eosin. Selected

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2 To whom requests for reprints should be addressed.
3 The abbreviations used are: AFB, aflatoxin B; AFP, a-fetoprotein; DMSO, dimethyl sulfoxide.

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specimens are also stained with periodic acid-Schiff, and frozen sections, when made, are stained with Oil Red O.

RESULTS

A total of 47 monkeys survived at least 2 months after the first dose of AFB, and 35 of these animals have thus far been necropsied. Twenty-two of the 35 monkeys showed no evidence of tumor upon histopathological examination of tissue following necropsy. These animals have been arbitrarily divided into 2 groups: those necropsied after receiving AFB, for less than 2 years; and those necropsied after receiving AFB, for longer than 2 years. Eleven monkeys survived between 2 and 21 months after initiation of AFB, treatment (Table 1). These monkeys received AFB, according to a variety of dosing schedules by either i.p. or p.o. (intubation or on vitamin sandwich) routes; total AFB, doses given the monkeys ranged from 0.08 to 28.88 mg. Seven of 11 monkeys in this group showed evidence of hepatic damage, with toxic hepatitis being the most frequent liver lesion found. However, the severity of the liver lesions did not appear to follow a close dose-response pattern. Thus, toxic hepatitis was detected upon histopathological examination of liver from 4 of 6 monkeys receiving less than 1 mg of AFB,; whereas no evidence of such damage was found in Monkeys 687I, 446F, and 519G given doses of 4.37, 5.00, and 28.88 mg of AFB, respectively. The toxic hepatitis noted in the monkeys showed a wide range of severity. Liver tissue from Monkey 689I (0.35 mg of AFB,) showed mild toxic hepatitis, whereas Monkey 532G developed severe toxic hepatitis after receiving a total AFB, dose of 0.64 mg. The liver lesions (Fig. 1) were characterized by necrosis of hepatic cells; those hepatocytes remaining showed swollen, deeply eosinophilic cytoplasm with frequent vacuoles. Lymphocytic infiltration around the necrotic cells was present, but there was very little formation of pseudotubules. Aside from liver lesions, the most common findings at necropsy of these monkeys were related to the respiratory system, and included bronchopneumonia and pulmonary congestion and edema.

Eleven monkeys were necropsied after receiving AFB, for periods ranging from 39 to 141 months and were found to be free of tumor (Table 2). Three of these animals were given doses i.p., 7 were given doses p.o., and one received AFB, both i.p. and p.o. Total AFB, doses given this group of animals ranged between 53.32 and 1368.69 mg. All but 2 of the monkeys showed histopathological evidence of liver damage, most frequently toxic hepatitis. In 2 cases with toxic hepatitis (Monkeys 587G and 527G), histopathological examination revealed the presence of hyperplastic liver nodules. Liver cirrhosis was found in 3 of the monkeys in this group, and in one case hyperplastic liver nodules were also present. Complications attributable to repeated i.p. injections (e.g., intestinal adhesions and obstruction) were noted in 3 of the 4 monkeys treated by this route.

To date, 13 of the 35 necropsied monkeys (37%) have developed malignant tumors following AFB, treatment (Table 3). This represents 26% of all monkeys receiving AFB,. In contrast, 68 nontreated breeder monkeys and vehicle-treated controls have died during this time period, of which 4 (5.9%) were found to have malignant tumors. The 4 monkeys (3 cases of malignant lymphoma in African green monkeys and one case of a gall bladder carcinoma in a 10-year-old rhesus monkey) represent 1.9% of all control and breeder monkeys maintained during this time in our monkey colony. Table 4 summarizes data for the 13 monkeys developing one or more malignant neoplasms after AFB, treatment.

The tumor-bearing monkeys received AFB, for periods of 47 to 147 months (average, 114 months). The total AFB, dose given these monkeys averaged 708.95 mg (range, 99.18 to 1354.24 mg). Five animals received AFB, both p.o. and i.p.; 8 received AFB, by the p.o. route only. The tumors developing in Monkeys 692I, 680H, and 454F have been described in detail in previous publications (3, 4) and are listed in the table for purposes of completeness only.

More than one primary tumor developed in 3 (Monkeys 590G, 683I, and 473F) of the 13 monkeys. Monkey 590G became inactive and developed a poor appetite approximately...
6 months prior to death. An elevation in the blood urea nitrogen (69.5 mg/100 ml) was noted on the day before the monkey died, but AFP levels were within normal limits. Histopathological examination of tissue from this animal revealed an undifferentiated neoplasm of the pancreas. The tumor was composed of a proliferation of spindle-shaped cells surrounding, but not destroying, acini and islets of Langerhans. In some areas, the neoplastic cells organized themselves to form small lumina destroying, acini and islets of Langerhans. In some areas, the neoplastic cells organized themselves to form small lumina throughout its lifetime. An osteogenic sarcoma of the distal radius developed in this monkey that was first observed as a hard and apparently painless swelling in the region of its wrist. This monkey began to lose weight approximately 4 months before the animal was sacrificed, the circumference of the leg was approximately 20 cm. The tumor mass at necropsy measured 9 x 10 cm and extended about 7 cm downward into the shaft of the tibia; its radiographic appearance resembled that of the tumor found in Monkey 683I. The histopathological examination showed to be a spindle-shaped neoplasm of the pancreas. The tumor was composed of sarcomatous elements with large irregular nuclei and prominent nucleoli and abundant osteoid formation (Fig. 7). The tumor invaded the bone marrow and penetrated the periosteum and soft tissue of the leg, but no distant metastases were found. Sections of liver from this monkey showed toxic hepatitis.

The second monkey (Monkey 683I) in which 2 primary tumors were diagnosed began to lose weight 20 months prior to diagnosis of tumor, but AFP levels remained negative throughout its lifetime. An osteogenic sarcoma of the distal radius developed in this monkey that was first observed as a hard and apparently painless swelling in the region of its wrist. The tumor grew rapidly, tripling in size in the 1-month period between the time it was first noted and the time the animal was sacrificed. Fig. 3 shows the radiographic appearance of the tumor. Necropsy and histological examination of tissue from this animal revealed an undifferentiated neoplasm of the pancreas. The tumor was composed of a proliferation of spindle-shaped cells surrounding, but not destroying, acini and islets of Langerhans. In some areas, the neoplastic cells organized themselves to form small lumina throughout its lifetime. An osteogenic sarcoma of the distal radius developed in this monkey that was first observed as a hard and apparently painless swelling in the region of its wrist. This monkey began to lose weight approximately 4 months before the animal was sacrificed, the circumference of the leg was approximately 20 cm. The tumor mass at necropsy measured 9 x 10 cm and extended about 7 cm downward into the shaft of the tibia; its radiographic appearance resembled that of the tumor found in Monkey 683I. The histopathological examination showed to be a spindle-shaped neoplasm of the pancreas. The tumor was composed of sarcomatous elements with large irregular nuclei and prominent nucleoli and abundant osteoid formation (Fig. 7). The tumor invaded the bone marrow and penetrated the periosteum and soft tissue of the leg, but no distant metastases were found. Sections of liver from this monkey showed toxic hepatitis.

The third monkey with 2 primary tumors, Monkey 473F, was noted to be pale, inactive, and without appetite and to have a markedly distended abdomen approximately 12.5 years after the first AFB dose. Over the next 4 days, approximately 1 liter of ascites fluid was removed from its peritoneal cavity. Laparotomy on the day prior to sacrifice revealed tumor in several lobes of the liver, although serum AFP levels were within normal limits. Two apparently independent tumors were diagnosed in this animal. One tumor was a well-differentiated adenocarcinoma of the hepatic duct (Fig. 6); this tumor invaded the liver parenchyma (Fig. 5) and the portal vein and had metastasized to numerous locations including the lungs, omentum, peritoneum, broad ligament of the uterus, and the urinary bladder serosa. The other primary tumor was an adenocarcinoma of the pancreatic duct (Fig. 6), which had invaded the pancreatic parenchyma and had permeated the perineural lymphatics.

The remainder of the monkeys developed single malignant neoplasms, all but one of which involved the hepatobiliary system or the pancreas. The exception was Monkey 582G. This monkey began to lose weight approximately 4 months before a swelling around its right knee was observed. One day before the animal was sacrificed, the circumference of the knee was approximately 20 cm. The tumor mass at necropsy measured 9 x 10 cm and extended about 7 cm downward into the shaft of the tibia; its radiographic appearance resembled that of the tumor found in Monkey 683I. The histopathological appearance of the tumor was compatible with a diagnosis of osteogenic sarcoma; the tumor was composed of sarcomatous elements with large irregular nuclei and prominent nucleoli and abundant osteoid formation (Fig. 7). The tumor invaded the bone marrow and penetrated the periosteum and soft tissue of the leg, but no distant metastases were found. Sections of liver from this monkey showed toxic hepatitis.

Monkeys 518G, 535G, 498F, 479F, 471F, and 374E developed malignant tumors of the liver, pancreas, gall bladder, or bile ducts, and, except in Monkey 471F, all tumors were accompanied by severe jaundice. Monkey 518G became jaundiced 1 day before it was sacrificed. A laparotomy had been performed approximately 2 years before its death because of an elevation in serum AFP level (120 ng/ml), but no tumor was grossly evident. The serum AFP level was 60 ng/ml 5 months later and at the time of sacrifice was within normal limits. At necropsy, a firm white mass was noted above the gall bladder which histopathological examination showed to be a spindle-
2. or 5 doses a week on alternate weeks. Dosing began within 9 days of birth for all monkeys.

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A second laparotomy performed 9 months later again revealed liver cirrhosis and a ductal papilloma of the breast. Differentiated adenocarcinoma of the cystic duct with extension to other extrahepatic bile ducts, liver parenchyma, pancreas, and pancreatic ducts. Additional findings included liver cirrhosis and a ductal papilloma of the breast.

An elevation in the serum AFP level (450 ng/ml) noted 112 days prior to sacrifice, the animal developed severe jaundice. At necropsy, tumor almost completely replacing the liver was found, which corresponded histologically to an hemangioendothelial sarcoma; multiple tumor metastases were found in the lungs. Sections of liver not involved with tumor showed marked toxic hepatitis.

One week before the death of Monkey 471F, the animal was noted to be inactive and to have a poor appetite although hematology and clinical chemistry values were within normal limits. Histological examination of tissue from this animal revealed an hemangioendothelial sarcoma of the liver with multiple metastases to the lung; areas of liver not involved with tumor showed micronodular cirrhosis with severe toxic hepatitis.

Table 4
Monkeys developing tumors after AFB treatment

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Species</th>
<th>Sex</th>
<th>Mos. on AFB</th>
<th>Route</th>
<th>Total AFB, dose (mg)</th>
<th>Tumors diagnosed</th>
<th>Other findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>692F</td>
<td>Rh</td>
<td>F</td>
<td>47</td>
<td>i.p.</td>
<td>99.18</td>
<td>Moderately well-differentiated hepatocellular carcinoma with invasion of stomach and lung metastases</td>
<td>Toxic hepatitis</td>
</tr>
<tr>
<td>680H</td>
<td>Rh</td>
<td>M</td>
<td>51</td>
<td>i.p.</td>
<td>119.44</td>
<td>Hemangioendothelial sarcoma of liver</td>
<td>Portal cirrhosis</td>
</tr>
<tr>
<td>590G</td>
<td>Rh</td>
<td>M</td>
<td>107</td>
<td>i.p.</td>
<td>291.83</td>
<td>Undifferentiated neoplasm of pancreas; multiple intramucosal Grade I papillary bladder carcinomas</td>
<td>Micronodular cirrhosis</td>
</tr>
<tr>
<td>683I</td>
<td>Rh</td>
<td>F</td>
<td>117</td>
<td>i.p.</td>
<td>353.28</td>
<td>Osteogenic sarcoma of radius; moderately well-differentiated adenocarcinoma of common bile duct with extension to gall bladder and liver hilum; adenocarcinoma of pancreatic ducts with invasion of parenchyma and lymph node; metastatic adenocarcinoma in soft tissues around vertebral column and ribs</td>
<td>Toxic hepatitis</td>
</tr>
<tr>
<td>582G</td>
<td>Cy</td>
<td>M</td>
<td>115</td>
<td>i.p.</td>
<td>411.52</td>
<td>Osteogenic sarcoma of tibia with invasion of periosteum and soft tissues of leg</td>
<td>Toxic hepatitis</td>
</tr>
<tr>
<td>518G</td>
<td>Cy</td>
<td>M</td>
<td>135</td>
<td>p.o.</td>
<td>515.49</td>
<td>Spindle-cell carcinoma of hepatic and common bile ducts</td>
<td>Cirrhosis</td>
</tr>
<tr>
<td>535G</td>
<td>Cy</td>
<td>F</td>
<td>135</td>
<td>p.o.</td>
<td>635.50</td>
<td>Well-differentiated adenocarcinoma of cystic duct with extension to other extrahepatic bile ducts, liver parenchyma, pancreas, and pancreatic ducts</td>
<td>Cirrhosis; ductal papilloma of breast</td>
</tr>
<tr>
<td>454F</td>
<td>Rh</td>
<td>F</td>
<td>74</td>
<td>p.o.</td>
<td>856.76</td>
<td>Anaplastic hepatocellular carcinoma</td>
<td>Focal necrosis and microabscesses of liver</td>
</tr>
<tr>
<td>498F</td>
<td>Cy</td>
<td>F</td>
<td>145</td>
<td>p.o.</td>
<td>1076.42</td>
<td>Hemangioendothelial sarcoma of liver with multiple pulmonary metastases</td>
<td>Extensive focal necrosis of liver</td>
</tr>
<tr>
<td>473F</td>
<td>Rh</td>
<td>F</td>
<td>142</td>
<td>p.o.</td>
<td>1103.00</td>
<td>Adenocarcinoma of hepatic duct invading liver parenchyma and portal vein with metastases to lungs, omentum, peritoneum, broad ligament, and bladder serosa; pancreatic duct adenocarcinoma with invasion of the parenchyma and lymphatic permeation</td>
<td>Extensive focal necrosis of liver</td>
</tr>
<tr>
<td>471F</td>
<td>Rh</td>
<td>F</td>
<td>147</td>
<td>p.o.</td>
<td>1147.36</td>
<td>Hemangioendothelial sarcoma of liver with pulmonary metastases</td>
<td>Micronodular cirrhosis with severe toxic hepatitis; pelvic endometriosis</td>
</tr>
<tr>
<td>479F</td>
<td>Rh</td>
<td>M</td>
<td>130</td>
<td>p.o.</td>
<td>1252.28</td>
<td>Well-differentiated gall bladder adenocarcinoma with invasion of liver and lung metastases</td>
<td>Toxic hepatitis</td>
</tr>
<tr>
<td>374E</td>
<td>Rh</td>
<td>F</td>
<td>136</td>
<td>p.o.</td>
<td>1354.24</td>
<td>Well-differentiated adenocarcinoma of common bile duct and ampulla of Vater with invasion of pancreas</td>
<td>Focal necrosis of liver</td>
</tr>
</tbody>
</table>

*a Rh, rhesus; Gr, African green; Cy, cynomolgus.*

cell carcinoma of the hepatic and common bile ducts. The poorly differentiated tumor invaded the wall of the common bile duct and projected in a polypoid manner into the lumen of some of the large bile ducts. It was composed primarily of undifferentiated spindle cells with occasional glandular differentiation (Fig. 8). Evidence of cirrhosis was also found in sections of liver from this animal (Fig. 9).

Monkey 535G developed marked jaundice 10 days before it was sacrificed; at necropsy, the neck of the gall bladder was found to be almost completely obstructed by dense white tissue which histopathological examination revealed to be a well-differentiated adenocarcinoma of the cystic duct with extension to the other extrahepatic bile ducts, the liver parenchyma, the pancreas, and the pancreatic ducts. Additional findings included liver cirrhosis and a ductal papilloma of the breast.

An elevation in the serum AFP level (450 ng/ml) noted 112 months after Monkey 498F received the first dose of AFB, prompted a laparotomy, but no evidence of tumor was found. A second laparotomy performed 9 months later again revealed no evidence of gross tumor. The animal developed a poor appetite and began losing weight 2 months before it was sacrificed, at which time AFP levels were within normal limits. Two days prior to sacrifice, the animal developed severe jaundice. At necropsy, tumor almost completely replacing the liver was found, which corresponded histologically to an hemangioendothelial sarcoma; multiple tumor metastases were found in the lungs. Sections of liver not involved with tumor showed marked toxic hepatitis.

One week before the death of Monkey 471F, the animal was noted to be inactive and to have a poor appetite although hematology and clinical chemistry values were within normal limits. Histological examination of tissue from this animal revealed an hemangioendothelial sarcoma of the liver with multiple metastases to the lung; areas of liver not involved with tumor showed micronodular cirrhosis with severe toxic hepatitis.

Monkey 479F became jaundiced about 2 months before its death, but AFP levels remained within normal limits throughout its life. A laparotomy was performed 6 years after initiation of AFB treatment, but no gross evidence of tumor was present. At necropsy of this animal, however, a mass of hard white tissue was noted in the region of the gall bladder, which was
classified as a well-differentiated gall bladder adenocarcinoma (Fig. 10). The tumor had invaded the liver parenchyma and had metastasized to the lungs. Sections of liver from areas not involved with tumor showed toxic hepatitis.

Liver biopsies performed 20, 52, and 85 months after Monkey 374E was given the first dose of AFB, failed to yield evidence of tumor, although toxic hepatitis was evident in sections of liver taken at the 2 later biopsies. AFB levels remained within normal limits throughout the life of this animal. Approximately 5 months before it was sacrificed, its body weight decreased from 8.6 kg to a terminal value of 5.7 kg. Marked jaundice developed, and a laparotomy was performed one week before sacrifice. Findings included dilation of the common bile duct, a heavily scarred gall bladder, and a shrunken and abnormally shaped liver, but no gross evidence of tumor was observed. However, following necropsy and histopathological examination of tissue from this animal, adenocarcinoma of the common bile duct and ampulla of Vater (Fig. 11) was diagnosed. The tumor had invaded the head of the pancreas, but no distant metastases were found.

Twelve monkeys are currently receiving weekly doses of AFB, (0.2 mg/kg) by intubation (Table 5). The total AFB, dose ingested by these monkeys thus far averages 952.65 mg and ranges between 355.68 and 1377.24 mg. The monkeys have been receiving continuous treatment with AFB, for periods ranging from 138 to 150 months (average, 142 months). All but 2 monkeys have received total doses of AFB, exceeding the average total dose given the 13 monkeys which developed malignant tumors; none of the animals has developed clinical signs of illness.

Thus, 13 of 47 Old World monkeys treated with AFB, have developed a total of 17 malignant neoplasms. These tumors included: 5 primary liver tumors (2 hepatocellular carcinomas and 3 hemangiendothelial sarcomas); one gall bladder adenocarcinoma; 5 carcinomas of the cystic, hepatic, and/or common bile ducts (one spindle-cell carcinoma, and 4 adenocarcinomas); 3 tumors of the pancreas or its ducts (one undifferentiated neoplasm and 2 adenocarcinomas); 2 osteogenic sarcomas; and one papillary Grade I carcinoma of the urinary bladder.

No clear pattern emerged when tumor type was analyzed with regard to sex, subspecies, route of administration, or dose of AFB, probably because of the relatively small numbers of tumor-bearing animals. The tumors developed in 8 of 22 (36%) necropsied rhesus monkeys, 4 of 11 (36%) necropsied cynomolgus monkeys, and in one of 2 (50%) necropsied African green monkeys. Evaluation of tumor incidence as a function of subspecies by \( \chi^2 \) analysis failed to show significant differences \((p > 0.9)\) in tumor incidence among the 3 groups. Five of 16 (31%) necropsied female monkeys and 8 of 19 (42%) necropsied male monkeys developed tumors; \( \chi^2 \) analysis of tumor incidence in males and females failed to reveal a statistically significant \((p > 0.30)\) sex difference. No tumors have developed in monkeys receiving AFB, by i.p. injection, whereas 8 of 21 (38%) necropsied monkeys treated p.o. developed tumors, as have 5 of 6 (83%) of animals treated both p.o. and i.p. Evaluation of tumor incidence as a function of treatment route revealed a significantly higher \((p < 0.01)\) tumor incidence in monkeys receiving AFB, both p.o. and i.p.

The monkeys developing tumor showed individual variation with regard to latent period and cumulative dose ingested.

Although the average AFB, dose administered to the monkeys was 708.95 mg, one monkey developed tumor after receiving only 99.18 mg of compound. All but 2 of the monkeys without tumor receiving AFB, longer than 2 years had ingested in excess of 99 mg of compound, and 4 of these had received total AFB, doses exceeding the average dose given the tumor-bearing animals. Furthermore, although the dosing interval for monkeys developing tumors averaged 114 months, the first AFB,,-induced tumor appeared after only 47 months of treatment.

**DISCUSSION**

AFB, is hepatotoxic and carcinogenic in many species of laboratory animals, including nonhuman primates. However, a considerable amount of species variation exists with regard to susceptibility to the carcinogenic and other adverse effects of aflatoxins. The mouse appears to be relatively resistant to the hepatotoxic and carcinogenic effects of aflatoxins (19, 26), whereas the effective hepatocarcinogenic dose of AFB, in rats has been estimated to be 50 \( \mu \)g/kg (9). The rainbow trout seems markedly more sensitive than do rodents to the hepatocarcinogenic effects of aflatoxins, inasmuch as doses of 0.2 to 2.0 \( \mu \)g AFB, per kg induce a high incidence of liver tumors in this species (6, 7).

Information has begun to accumulate on the hepatotoxicity and carcinogenicity of aflatoxins in human and nonhuman primates. The studies of Reddy *et al.* (28) have shown that the prosimian primate *Tupaia glis*, or tree shrew, is extremely sensitive to AFB,,-induced hepatotoxicity and hepatocarcinogenicity. All 6 females and 3 of 6 males developed hepatocellular carcinomas between 74 and 172 months after the first dose of AFB,; the cumulative doses given the animals developing tumors ranged between 24 and 66 mg. The predominant lesions found at necropsy were related to the liver, and no changes in sections from the biliary tract or pancreas were described. Liver changes noted ranged from diffuse fatty metaplasia of liver parenchyma in animals that died early in the course of the experiment to severe postnecrotic scarring, stellate scarring of isolated portal tracts accompanied by oval cell proliferation, necrosis of isolated liver cells in periportal liver cords, and mononuclear cell infiltrates in animals surviving up to 172 weeks. Considerable individual variation existed with regard to the response of the tree shrew liver to AFB, and the
degree of liver damage did not correspond to the cumulative
dose of carcinogen ingested (28).

The first reports on the effects of aflatoxins in higher primates
(Old World monkeys) described results from acute and sub-
acute feeding studies which were probably terminated too early
for a carcinogenic effect to become apparent. The histopath-
ological lesions noted in liver from treated animals were widely
variable in severity and, as was the case with tree shrews,
could not be correlated with cumulative aflatoxin dose. Unlike
the tree shrew, however, proliferation of bile duct epithelium
was a consistent finding in all of the studies. Thus, Madhavan
et al. (18) gave a group of young rhesus monkeys daily doses
of aflatoxin (0.5 and 1.0 mg) and found severe fatty metamor-
phosis and portal fibrosis in the animals within 4 weeks of the
first dose of aflatoxin. A striking proliferation of ductules was
noted in the portal tracts, along with fibrosis and chronic
inflammatory cells. The histogenesis of the hepatic fibrosis was
explained in its entirety on the basis of portal inflammation, bile
duct proliferation, and periductular fibrosis. In a study reported
by Cuthbertson et al. (10), a group of cynomolgus monkeys
was fed a diet of groundnut meal containing aflatoxins for up
to 3 years. A variety of hepatic lesions was noted at necropsy,
including central zonal liver cell necrosis or degeneration ac-
companied by liver cell regeneration and bile duct and fibro-
blastic proliferation. Liver cell atypia and fatty change was also
found in the animals. Similar observations were made by Deo
et al. (11), who fed rhesus monkeys aflatoxins for periods up
to 2 years. A triad of histological changes consisting of large
hyperchromatic liver cells, bile duct epithelial and stromal
proliferation, and condensation around proliferated ductular
cells was a frequent finding. However, no changes were noted
in other animals given identical quantities of aflatoxin for the
same length of time. Bourgeois et al. (8) evaluated the histo-
logical changes developing in the livers of monkeys killed one
week after a single dose of AFB1 (0.5 to 40.5 mg/kg). At doses
exceeding 1.5 mg/kg, the liver changes they found included
fatty degeneration of hepatocytes, decreased liver glycogen,
liver cell necrosis, and hyperplasia of the bile duct epithelium.
Similar histological changes were found in the monkeys in the
present study, although considerable individual variation in
sensitivity to AFB1 was noted. In the tumor-free monkeys ne-
cropsied thus far, the hepatic lesions have not correlated well
with the total AFB1 dose administered. Thus, toxic hepatitis
was present in liver from some monkeys receiving a total AFB1
dose of less than 1 mg, whereas no liver lesions were noted in
other animals which had ingested as much as 515 mg of AFB1.

Studies in which aflatoxin has been administered to Old
World monkeys for relatively prolonged periods indicate that it
is a carcinogen as well as an hepatotoxin in higher primates.
Thus, Tilak (30) reported that a female rhesus monkey de-
veloped a cholangiocarcinoma of the intrahepatic bile ducts after
receiving a mixture of aflatoxins for 5.5 years. A male rhesus
monkey developed a primary hepatocellular carcinoma after
prolonged treatment with a mixture of aflatoxins (12).

Some tentative conclusions can now be drawn as to the
carcinogenicity and hepatotoxicity of aflatoxins in various ani-
mal species. First, although the degree of liver damage in rats,
rainbow trout, and marmosets appears to closely parallel the
cumulative dose of aflatoxin received (17, 20, 21), this does
not seem to be the case in either tree shrews or Old World
monkeys. Second, the type of histological lesions induced by
aflatoxins appears to be dependent upon many variables, in-
cluding the species. Thus, aflatoxins induce hepatocellular
damage and primary liver carcinomas in rainbow trout, rats,
and tree shrews, whereas the most striking and consistent
lesions in Old World monkeys and in humans appear to be
related to the biliary system. In many species of domestic
animal, the subacute toxicity of aflatoxins is most consistently
manifested as proliferation of the bile duct epithelium (32), and
the duckling is particularly prone to develop this lesion follow-
ing acute exposure to aflatoxins; in fact, the sensitivity of
ducking bile duct epithelium provided the basis for bioassays
of aflatoxin contamination of foodstuffs (5, 22). Bile duct lesions
are also frequently found in cases of human aflatoxicosis.

Histopathological examination of liver taken at autopsy of
persons following an outbreak of aflatoxicosis in Western India
revealed pathological changes similar to those in the monkeys
in the present study, particularly bile duct proliferation with
periductilar fibrosis (14). The clinical features of the hepatitis
that developed in these patients prior to autopsy included
portal hypertension, jaundice, and rapidly developing ascites,
and they were associated with a high mortality rate. Although
the first 3 tumors that arose in our AFB1-treated monkeys were
primary liver tumors (3, 4), 9 of the 14 tumors that subsequently
emerged were malignant tumors of the biliary system or pan-
creas. The monkeys developing these tumors showed many of
the clinical features of the hepatitis developing in patients
exposed to aflatoxins, including jaundice and rapidly develop-
ing ascites.

Studies in rats have indicated a sex difference with regard to
tumor induction by aflatoxins, with females showing greater
resistance to hepatocarcinogenesis than males (33). This does
not appear to be the case with either the tree shrew or the Old
World monkey. In the present study, no sex differences were
noted with regard to incidence or severity of liver lesions in
fume-free monkeys. Furthermore, neither the overall incidence
of tumors nor the incidence of liver, biliary, or pancreatic
tumors appeared to be sex related.

Thus, the response of the rat to aflatoxins appears to be
somewhat different from that of the duckling, nonhuman pri-
mates, and humans. Furthermore, studies from this laboratory
(2) have shown that prosimian primates, such as the tree shrew
and galago, tend to resemble rats more closely than higher
primates, such as Old World monkeys, with regard to response
to some chemical carcinogens (e.g., polycyclic hydrocarbons).
The present study provides further evidence for this, since a
preponderance of the tumors induced in Old World monkeys
by AFB1 were carcinomas of the biliary tract and pancreas, in
contrast to the primary liver carcinomas induced in tree shrews
and rats.

The development of the 2 osteogenic sarcomas in the AFB1-
treated monkeys was an interesting finding. To our knowledge,
aflatoxins have not been associated with this type of tumor in
any other experimental animal or in humans. No cases of
spontaneous osteogenic sarcomas have appeared in our mon-
key colony over the past 16 years, although 5 cases of spono-
taneous osteogenic sarcomas have been reported in other
colonies of nonhuman primates (23). However, we recently
reported finding osteogenic sarcomas in 2 monkeys receiving
long-term treatment with procarbazine, a known chemical car-
cinogen (29); the sarcomas developed in the jaw and humerus
of a female cynomolgous and a female rhesus monkey, respec-
tively. Thus, it appears that this relatively rare type of tumor can be induced in nonhuman primates by chemical carcinogens as well as by irradiation (15, 16, 24, 25, 31).

The present study clearly demonstrates that AFB\textsubscript{1} is a hepatotoxin and carcinogen in Old World monkeys. The results not only lend additional support to the hypothesis that AFB\textsubscript{1} is an hepatocarcinogen in humans but also raise the possibility that this chemical may play a role in the etiology of human pancreatic and gall bladder carcinoma and osteogenic sarcoma.

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REFERENCES

Fig. 1. Liver from Monkey 532G, showing severe toxic hepatitis. H & E, x 100.

Fig. 2. Intramucosal Grade I papillary carcinomas of the urinary bladder from Monkey 590G. H & E, x 40.

Fig. 3. X-Ray of osteogenic sarcoma of the distal radius that developed in Monkey 6831.

Fig. 4. Adenocarcinoma of hepatic duct developing in Monkey 473F. H & E, x 40.
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Fig. 5. Bile duct adenocarcinoma invading liver parenchyma in Monkey 473F. H & E, × 100.
Fig. 6. Pancreatic duct adenocarcinoma invading the parenchyma in Monkey 473F. H & E, × 100.
Fig. 7. Osteogenic sarcoma of the tibia of Monkey 582G. H & E, × 100.
Fig. 8. Spindle-cell carcinoma of the hepatic and common bile ducts of Monkey 518G. H & E, × 100.
Fig. 9. Liver from Monkey 518G, showing cirrhosis of the liver. H & E, x 40.

Fig. 10. Well-differentiated gall bladder adenocarcinoma in Monkey 479F. H & E, x 40.

Fig. 11. Adenocarcinoma of the common bile duct and ampulla of Vater found in Monkey 374E. H & E, x 40.
Induction of Osteogenic Sarcomas and Tumors of the Hepatobiliary System in Nonhuman Primates with Aflatoxin B₁

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