Induction of Liver Tumors by Aflatoxin B$_1$ in the Tree Shrew (Tupaia glis), a Nonhuman Primate

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SUMMARY

The epidemiological studies suggest that aflatoxins, the toxic metabolites of the ubiquitous mold Aspergillus flavus, may play a significant role in the evolution of hepatocellular carcinoma in man in certain geographic areas of the world. To ascertain their carcinogenicity in nonhuman primates, we have administered highly purified aflatoxin B$_1$, intermittently in the diet at 2 ppm, to 10 female and 8 male tree shrews. The tree shrew (Tupaia glis) is a nonhuman primate occurring throughout Southeast Asia which can be reared easily in captivity. Of 12 animals that survived, 6 of 6 female shrews. The tree shrew (Tupaia glis) is a nonhuman primate tentently in the diet at 2 ppm, to 10 female and 8 male tree shrews, the liver tumors were associated with severe post necrotic scarring; in the other 7 tumor-bearing livers, only mild to moderate portal fibrosis was encountered. This individual variation in hepatocellular response and in the amount of aflatoxin B$_1$ consumed by these animals ranged from 24 to 66 mg. The development of liver tumors did not follow a specific pattern; considerable variation in hepatocellular carcinomas between 74 and 172 weeks after the beginning of the experiment. None of the 8 control animals developed liver cancers. The estimated total amount of aflatoxin B$_1$ consumed by these animals ranged from 24 to 66 mg. The development of liver tumors did not follow a specific pattern; considerable variation in hepatocellular responses to aflatoxin B$_1$ was noted in these animals. In 2 tree shrews, the liver tumors were associated with severe post necrotic scarring; in the other 7 tumor-bearing livers, only mild to moderate portal fibrosis was encountered. This individual variation in hepatocellular response and in the amount of aflatoxin B$_1$ required to induce hepatocellular carcinomas is attributed to inherent differences in the susceptibility within a given species of outbred animals and suggests extreme caution in proposing the "permissible" or "safe" levels of contamination of carcinogens in the food-stuffs.

INTRODUCTION

The aflatoxins are a group of metabolites elaborated by some strains of the mold Aspergillus flavus, which grows readily on peanuts and other grains, such as maize, stored in warm weather and humid conditions (12, 22, 27). These fungal metabolites exert pronounced hepatotoxic effects in a wide variety of laboratory and farm animals (15, 27). Aflatoxin B$_1$, the most potent of these toxins, produces hepatocellular carcinomas in rats, mice, ducks, ferrets, rainbow trout, and guppies (3, 8, 15, 16, 20). Also, available epidemiological data from various developing countries of the world reveal a definite correlation between the risk to man of developing acute or chronic liver disease, including the occurrence of primary hepatocellular carcinoma, and the degree to which food products are contaminated with aflatoxins (21, 26). This suggested relationship between the incidence of liver cancer and the amount of aflatoxin consumed with the contaminated diet (21, 26) may not be construed as unequivocal evidence of a cause-and-effect relationship, because the present levels of contamination may not necessarily reflect the extent of contamination that existed 5 to 20 years ago, i.e., the possible period of time required for the development of hepatocellular carcinoma in man. Also, at least 2 other factors, cirrhosis of the liver due to various etiological factors including malnutrition and high frequency of hepatitis Bs antigen are also frequently associated with primary cancer of the liver in man (9, 23). To our knowledge, no prospective statistical study of aflatoxin B$_1$ contamination of diet and incidence of hepatoma in man has been reported.

Despite the possible interplay of several factors, the circumstantial evidence regarding the role of the aflatoxins in the evolution of human liver disease is persuasive enough to investigate their carcinogenic effect in nonhuman primates, as the response of lower primates to environmental factors can be similar to that of humans (5). The available reports deal with the development of hepatocellular carcinoma in 2 rhesus monkeys (2, 7) and a cholangiocarcinoma in the 3rd (25), following long-term exposure to aflatoxin B$_1$. This communication deals with the aflatoxin B$_1$ induction of hepatocellular carcinomas in tree shrews (Tupaia glis), which are small squirrel-like mammals occurring throughout Southeast Asia and are regarded as primitive primates (14).

MATERIALS AND METHODS

Tree shrews (T. glis) weighing 95 to 140 g were obtained from Animal World Laboratories, Miami, Fla., and housed individually in stainless steel rat cages (24 cm high, 38 cm wide, 53 cm deep). A rectangular tube 25 cm long, 7.5 cm high, and 11 cm wide, covered with wire mesh at 1 end, was provided in each cage for these animals to hide in during the daytime because of their nocturnal habitat. The tubes also offered a convenient mechanism to restrain these fast-moving animals with a gloved hand for periodic examination and weekly weighing. Each cage was also provided with 2 porcelain dishes, 1 for the diet and the other for water. Twenty-six animals were divided into 2 groups. Group 1

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contained 10 females and 8 males that were given pure crystalline aflatoxin B1 (Makor Chemicals Ltd., Jerusalem, Israel), at a dietary concentration of 2 ppm. Group 2, composed of 3 females and 5 males, served as controls and these animals were fed the same diet as was Group 1 but lacking aflatoxin B1. The diet consisted of finely powdered Purina monkey chow (Ralston Purina Company, St. Louis, Mo.) mixed with freshly blended banana and orange squash. The administration of aflatoxin B1-containing diet to animals in Group 1 followed an on-and-off schedule which was adjusted periodically to ensure maximum survival. For convenience, only the schedule of aflatoxin feeding of tree shrews that developed liver tumors is depicted in Chart 1. The experiment was terminated after a total duration of 172 weeks. The daily consumption of diet by these tree shrews averaged 40 g. The estimated total intake of aflatoxin B1 by animals bearing hepatocellular carcinomas ranged from 24 to 66 mg.

Laparotomies were performed at selected intervals, under light methoxyflurane (Metofane; Pitman-Moore Div., Fort Washington, Pa.) anesthesia, and liver biopsies were obtained for light and electron microscopic examination. Autopsies were done when the animals were found moribund or dead. For light microscopy, tissues were fixed in neutral buffered formalin and embedded in paraffin, and all the sections were routinely stained with hematoxylin and eosin. When indicated selected sections were stained with Gomori's trichrome stain. The sections were examined with a Zeiss Ultraphot II microscope and were photographed with a green filter. Selected samples of liver biopsies and liver tumors were fixed for 1 to 2 hr in osmium tetroxide buffered with s-collidine to pH 7.4 at 0–4° and processed for electron microscopy.

RESULTS

The dietary intake as well as gain in body weight of tree shrews in both groups showed no appreciable differences.

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\[\text{ON AFLATOXIN DIET}\]

Chart 1. The schedule of aflatoxin B1 feeding of 3 male and 6 female tree shrews that developed hepatocellular carcinomas.

During the 1st 10 weeks of the experiment, the livers of aflatoxin-treated animals showed varying degree of fatty metamorphosis. The frequent finding in aflatoxin-fed animals (Group 1) that died within the 1st 40 weeks of the experiment was diffuse fatty metamorphosis of the liver parenchyma. The liver cells were studded with fat droplets which generally appeared as discrete, small cytoplasmic vacuoles. In addition to this microvesicular fat, some portions of hepatocyte cytoplasm displayed scattered large fat droplets. At subsequent intervals, the liver biopsies obtained at periodic laparotomies or tissue procured at autopsy from dead animals revealed different degrees of hepatic damage. The extent of hepatic alterations did not seem to correlate well with the amount of aflatoxin B1 consumed by these animals. This striking variation in the hepatic damage from animal to animal was pronounced after the 40th week of the experiment. In addition to the fatty change, which varied considerably in intensity throughout the experimental period, the other pertinent changes in the liver of affected animals consisted of 1 or more of the following: (a) the presence of scattered light- and dark-staining liver cells; (b) appearance of multiple, well-defined microscopic foci of hyperchromatic liver cells with deeply basophilic cytoplasm, displaying cellular pleomorphism and variation in nuclear size, including atypism; (c) distortion of normal lobular architecture resulting from regeneration of liver cells and collapse of reticular framework; and (d) isolated areas of degenerating hepatic parenchymal cells showing hydropic or feathery degeneration. In addition, stellate scarring of isolated portal tracts accompanied by oval cell proliferation, necrosis of isolated liver cells in perportal liver cell cords, and mononuclear cell infiltrates were encountered in 6 animals. Also, the livers of 2 tree shrews, killed at 74 and 75 weeks, revealed severe postnecrotic scarring.

Between 74 and 172 weeks of the experiment, 9 of 12 tree shrews that survived more than 74 weeks on the experiment developed liver tumors. These tumors occurred in 6 of 6 females (100%) at 74, 112, 124, 141, 169, and 172 weeks and in 3 of 6 males (50%) at 75, 147, and 169 weeks of the experiment. The schedule of aflatoxin B1 administration to tree shrews that developed liver tumors. (Chart 1) clearly indicates the wide variability in susceptibility of these wild outbred animals to a very potent hepatocarcinogen. The estimated consumption of aflatoxin B1 in animals that developed liver tumors varied from 24 to 66 mg. Grossly, the liver tumors appeared as either solitary or multiple, irregular, gray to brown nodules of varying size (Figs. 1 to 3), usually protruding from the liver surface and displaying prominent vascularity (Fig. 1). They measured from 0.2 to 3.5 cm in diameter and, on section, had a variegated appearance. Some large tumors revealed areas of hemorrhage and necrosis (Fig. 3). Several of these tumors were poorly demarcated from the surrounding liver, while others appeared somewhat well demarcated. In 2 tree shrews, the liver tumors were associated with extensive postnecrotic scarring of the remaining hepatic parenchyma, whereas, in other 7 tumor-bearing livers, only mild to moderate stellate fibrosis of portal tracts with variable degree of oval cell proliferation was encountered. Histologically, the liver tumors of all 9...
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tree shrews were well to poorly differentiated hepatocellular carcinomas (Figs. 4 to 9). Also, considerable variation in the cytological differentiation of different tumor nodules in a given liver was evident. The predominant pattern, in several small to medium-sized tumors, was that of a well-differentiated trabecular hepatoma (Figs. 4 and 5), although some small tumors were less well differentiated, composed of large polyhedral or pleomorphic cell type (Figs. 6 and 7). Much larger nodules were comprised of irregular masses of poorly differentiated or highly anaplastic cell types of hepatocellular carcinomas (Fig. 8). Because of the histological characteristics of these lesions and the occasional suggestion of microscopic vascular invasion within the liver (Fig. 9), these tumors were classified as liver cell carcinomas.

Six hepatic tumors were studied by electron microscopy. They differed considerably from one another in the degree of differentiation at the ultrastructural level. The electron microscopic appearance of a relatively well-differentiated trabecular hepatocellular carcinoma is illustrated in Fig. 10. In these tumor cells, the mitochondria, although numerous, appeared small; lysosomes, peroxisomes (microbodies), focal areas of autophagocytosis, and irregular strands of rough endoplasmic reticulum were also seen. Several peroxisomes contained a large nucleoid or crystalline core. The ultrastructure of moderately well-differentiated hepatocellular carcinomas is illustrated in Figs. 11 and 12. In contrast, the poorly differentiated hepatomas were characterized by scantiness of subcellular organelles (Fig. 13). Considerable variation in size of mitochondria was evident; some contained matrix striations. Nucleoid containing peroxisomes were rarely seen in poorly differentiated liver tumors (Fig. 13). The bile canaliculi were generally irregular and possessed few microvilli (Fig. 13). The tumor cell nuclei contained prominent nucleoli often showing microsegregation. Cytoplasmic invaginations into the nucleoplasm and lipid inclusions were also encountered in several nuclei of tumor cells. With the exception of multiple pulmonary adenomas in 1 animal, no other tumors were encountered in the aflatoxin B1-treated group. The control tree shrews, which were followed for 4 years, did not develop tumors of the liver or of other organs.

DISCUSSION

Spontaneous tumors of liver are rarely seen in subhuman primates (17). However, hepatocellular carcinomas of the liver are easily inducible in several species of monkeys by the administration of diethylnitrosamine, either in the diet or by i.p. injections (10). The effect of other potent chemical carcinogens such as azo dyes, acetylaminofluorene, urethan, and methyldioxynamethanol acetate on the liver of various nonhuman primates, over a relatively long period of time, was also investigated by O'Gara and Adamson (17) and Adamson et al. (1); but these compounds failed to induce liver tumors. Because of the possible implication of aflatoxin consumption in the high incidence of human liver cancer (16, 21, 27), several investigators have studied the acute and long-term effects of these fungal metabolites in rhesus and cynomolgus monkeys (4, 5, 24). Deo et al. (5) and Cuthbertson et al. (4) have fed aflatoxin to rhesus and cynomolgus monkeys, respectively, for periods of up to 30 months without obtaining liver tumors. Recently, however, Adamson et al. (2) observed a primary liver cell carcinoma in 1 rhesus monkey fed aflatoxin B1, for 6 years. Also, of the 2 rhesus monkeys treated with aflatoxin B1, for over 5 years at the National Institute of Nutrition, Hyderabad, India, one (a male) developed a hepatocellular carcinoma (7), while the other (a female) developed an intrahepatic cholangiocarcinoma (25).

The present studies clearly demonstrate that aflatoxin B1 is hepatotoxic and hepatocarcinogenic to another species of nonhuman primate, the tree shrew. The acute dose of aflatoxin B1, that is lethal to 50% of tree shrews, from preliminary experiments, has been estimated at 0.5 mg/kg. At 1 mg/kg, this agent produced severe acute fatty metamorphosis of the liver and marked cytoplasmic and nuclear changes at the ultrastructural level (J. K. Reddy, unpublished data). Dissociation of ribosomes from the endoplasmic reticulum membranes, mitochondrial damage, and segregation of nucleolar components into granular and fibrillar zones were seen in the liver cells between 12 and 24 hr following a single i.p. injection of aflatoxin B1 at 1 to 4 mg/kg (19). At a dietary concentration of 2 ppm, this naturally occurring hepatocarcinogen induced liver tumors in 6 female and 3 male tree shrews that survived long-term treatment. Two of these tumors were associated with extensive postnecrotic scarring of uninvolved liver, whereas the other tumors were in noncirrhotic livers arising as multicentric nodules. The tumors were composed of well to poorly differentiated hepatocellular carcinomatous cells, often with large, bizarre, hyperchromatic nuclei containing prominent nucleoli. Mitoses and tumor giant cells were frequently seen. Although distant metastases were not observed there was, however, evidence of tumor invasion within the intrahepatic vascular channels. Accordingly, these tumors are considered as hepatocellular carcinomas. The ultrastructural studies confirmed the liver cell origin of these tumors.

The sequential morphological changes indicated a marked variability in the response of tree shrew liver to aflatoxin; the degree of liver damage did not correspond to the cumulative dose of the carcinogen. This variability in liver changes is attributed to inherent differences in the susceptibility within a given species of outbred (wild) animals, because a more predictable and uniform response is likely to be elicited in highly inbred animals. Deo et al. (5) also observed considerable variation in liver changes in rhesus monkeys given identical quantity of aflatoxin over the same period of time. Some degree of variability in the response of cynomolgus monkeys to aflatoxin was also evident in the studies described by Cuthbertson et al. (4). However, in rats, rainbow trout, and marmosets, the degree of liver damage has been found to parallel closely the accumulated dose of this toxin (13, 15, 16).

The individual variations in hepatocellular response and in the amount of aflatoxin B1 required to induce liver tumors (estimated 24 to 66 mg) in the random-bred tree shrews suggest extreme caution in determining the “permissible” or “safe” levels of contamination of any toxin in the food stuffs.

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Although epidemiological studies have strongly suggested that aflatoxins may be responsible for human liver disease including the development of liver cancer, there have thus far been only few reports directly incriminating this toxin. Recently, Krishnamachari et al. (11) have reported an outbreak of a syndrome characterized by icterus, rapid onset of signs of portal hypertension, ascites, and a high mortality rate in a total of 397 persons who consumed maize contaminated heavily with A. flavus. Analysis of contaminated samples suggested that affected individuals could have consumed between 2 and 6 mg of aflatoxin daily over a period of 1 month. They also observed similar disease in dogs, which invariably shared the food of affected households, during the outbreak of this epidemic. This, coupled with the fact that the outbreak of this disease lasted only until the stock of spoiled maize was consumed, suggested that the disease is the direct result of aflatoxicosis. Also, the recent epidemiological studies of van Rensburg et al. (26), which deal with the primary liver cancer rate and aflatoxin intake in a high-cancer area, reveal a significant correlation between the level of aflatoxin consumption and the liver cancer rate. The foregoing studies as well as the fact that a broad spectrum of animals, including nonhuman primates, develop liver cancer lend further support to the contention that aflatoxin is hepatotoxic and hepatocarcinogenic to man. It is also likely that a concerted action of several factors, such as malnutrition, viral hepatitis, pyrrolizidine alkaloids, aflatoxins, and other yet unidentified factors might be responsible for the high incidence of liver cancer in certain geographic areas (9, 13, 18, 23).

This study also emphasizes the usefulness of the tree shrew for the long-term carcinogenic studies. Relatively large numbers of these primates are easily maintained in a limited space at a reasonable cost for several years. Tree shrew maintenance costs are 10 to 15 times cheaper than that of other primates. At present these primitive primates are easily obtainable, whereas other Old and New World monkeys are difficult to obtain because of restrictions imposed by exporting nations. Furthermore, successful colonization of Tupaiia under standard laboratory conditions is possible; with a gestation period of nearly 45 days, as many as 5 litters/year can be expected (6, 14). Accordingly, the tree shrew can be reared in large numbers in captivity and may prove to be a suitable nonhuman primate for tests involving potential chemical carcinogens and for evaluation of "safe" levels of known hepatocarcinogens.

ACKNOWLEDGMENTS

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REFERENCES

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Fig. 1. Large solitary liver tumor in a male tree shrew killed at 147 weeks on the experiment. Estimated total consumption of aflatoxin B1 was 50 mg.

Fig. 2. Multiple liver tumors in a female tree shrew killed at 112 weeks. Estimated total consumption of aflatoxin B1 was 24 mg.

Fig. 3. Cross-section of the liver tumor from a female tree shrew that died at 169 weeks. Total aflatoxin B1 consumption was 66 mg. Areas of hemorrhage and necrosis are seen.

Figs. 4 to 9. All sections were stained with hematoxylin and eosin.

Fig. 4. Area of trabecular hepatocellular carcinoma from a male tree shrew. × 180.

Fig. 5. Portion of another trabecular hepatocellular carcinoma, with several large tumor cells with bizarre nuclei. × 175.

Figs. 6 and 7. Moderately differentiated hepatocellular carcinomas in a female tree shrew contain large polyhedral tumor cells with abundant cytoplasm. Cytoplasmic vacuoles are seen in some tumor cells, probably represent lipid. × 180.

Fig. 8. Highly anaplastic hepatocellular carcinoma. Note the marked variation in tumor cell size. Several multinucleate giant cells are also present. × 180.

Fig. 9. Invasion of vessel wall by hepatocellular carcinoma. × 150.

Figs. 10 and 11. Electron microscopic appearance of aflatoxin B1-induced trabecular hepatocellular carcinoma in the tree shrew. Mitochondria are small; their number varies considerably from cell to cell. Rough endoplasmic reticulum strands are irregular. Autophagic vacuoles (Av), peroxisomes (P) with prominent nucleoids, lipid droplets (Li) are present. The cell junctions are irregular; also note abortive formation of bile canaliculi (Bc). N, nucleus. Fig. 10, × 6,200; Fig. 11, × 8,500.

Fig. 12. Portion of a tumor cell from a moderately differentiated hepatocellular carcinoma. Mitochondrial pleomorphism is evident. The rough endoplasmic reticulum channels are highly irregular in their distribution. × 8,500.

Fig. 13. Portions of 4 tumor cells from a poorly differentiated hepatocellular carcinoma. Note the paucity of cytoplasmic organelles. The bile canaliculi (Bc) are highly irregular and contain only an occasional microvillus. Nucleoid containing peroxisomes (P) were rarely seen in these tumor cells. × 28,000.
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