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Carcinogenesis in Rats by Aflatoxins B_1, G_1, and B_2 [1]

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SUMMARY

Aflatoxins B_1, G_1, and B_2 of high purity have been prepared from a crude mixture of aflatoxins and have been tested by long-term feeding in drinking water to rats, at concentrations of 1 μg/ml and 3 μg/ml. Aflatoxin B_1 produced liver tumors in 19 of 30 rats given a total dose of 2 mg each; 3 of 10 animals receiving a total dose of 1 mg developed liver tumors. Aflatoxin G_1 gave rise to liver tumors in 3 of 30 animals given a total dose of 2 mg and to 1 liver tumor in 10 animals given 1 mg each. However, of 26 rats receiving a total of 6 mg aflatoxin G_1, 21 animals developed liver tumors, and 6 animals had kidney tumors. A number of animals receiving 2 mg of aflatoxin G_1 also had kidney tumors. There was no sex difference in the incidence of liver tumors, but kidney tumors were seen only in males. The liver tumors were almost all hepatocellular carcinomas. No liver tumors were seen in 10 rats receiving a total dose of 1 mg aflatoxin B_2.

INTRODUCTION

The aflatoxins are now recognized as being extremely effective hepatocarcinogens to the rat, both when fed as naturally contaminated peanut meal (3, 10) or as mixed aflatoxins (2). At present, of the four major aflatoxins described, only pure aflatoxin B_1 has been adequately tested and shown to be carcinogenic (16). It is the purpose of this paper to describe the carcinogenicity of pure aflatoxins B_1 and G_1 and to present a preliminary experiment with aflatoxin B_2.

MATERIALS AND METHODS

A crude mixture of crystalline aflatoxins was obtained from Dr. K. Sargeant, Porton, England. Analysis of thin-layer chromatography (TLC) plates of silica gel (8) showed that it contained 44% aflatoxin B_1, 46% G_1, 6% B_2, and 3% G_2. The mixture was resolved into its components by repeated chromatography on TLC plates of 1-mm thick silica gel G with a mixture of chloroform:ether:acetic acid (2:2:1) as the developing solvent. About 20 mg of the mixed aflatoxins could be applied to a single 20 x 20 cm plate without overloading. The four main fluorescent bands were separately scraped from the plate and combined with corresponding bands from other plates. The adsorbed compounds were eluted three times with chloroform:ethanol (1:3), and the clear solution (obtained by filtration or centrifugation) was evaporated to dryness in a rotary evaporator. The composition of each fraction was determined by analysis of a small quantity on a 5 x 20 cm plate in the same solvent system (8). The fractions were rechromatographed in the same system until each contained negligible material other than the one component. The eluted material at this stage was, after removal of the solvent, a solid consisting of one of the aflatoxins together with soluble components of the silica gel adsorbent. These latter were removed by dissolution of the material in a small volume of warm chloroform, centrifugation (to sediment the inorganic material), and crystallization of the aflatoxin by addition of methanol to the warm chloroform solution followed by cooling.

In this way, from approximately 1 gm of aflatoxin mixture, about 250 mg each of aflatoxins B_1 and G_1 and a little over 20 mg of aflatoxin B_2 were obtained. The losses were considerable, and an insufficient amount of aflatoxin G_2 was obtained for any biologic test. Each of the crystalline products was assayed, and all were more than 96% pure, as determined by thin-layer chromatography of approximately 1 mg and estimation of the amount of aflatoxin in each fluorescent band by absorption spectrometry (8).

Randomly bred male and female MRC rats, 8–9 weeks old at the start of the experiment, were housed in plastic cages and fed Rockland food pellets and water *ad libitum*. The aflatoxins were administered in the drinking water using dark bottles at night to avoid photolysis (8). One hundred ml were offered to each cage of 5 animals for 5 nights each week, and any residual solution was measured and discarded on the following morning. Usually the whole 100 ml were consumed.

The solutions were prepared by saturating approximately 1 liter of distilled water with the aflatoxin by warming a suspension of a few milligrams of the solid for an hour over a steam bath. The suspension was filtered, and an absorption spectrum of the clear solution was taken. Alternatively the aflatoxin was dissolved in a few drops of dimethylformamide

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which was then added to the water. The concentration of aflatoxin was determined from its absorbance at 362–365 mμ according to the formula 1·0 absorbance = 14.3 μg/ml of B_1, 20.4 μg/ml of G_1, and 21.4 μg/ml of B_2. The parent solution was diluted with the required amount of water to a final concentration of 1 μg/ml or 3 μg/ml which was fed to the animals. The differing treatments of the animals are listed in Table 1. At the conclusion of the treatment the animals were observed until they were in poor condition or, in some cases, were found dead. No attempt was made to follow progressively any lesions induced by the treatment.

RESULTS

The animals were in good condition throughout the administration of the aflatoxins. During this period the weight gain of the treated animals was similar to that of the untreated controls. The survival of the rats is shown in Table 1. It can be seen that untreated animals survived better than the treated animals. The last controls were killed between the 100th week and the 105th week of the experiment, even though they were in good condition.

The incidence of neoplasms in the groups is given in Table 2. All but a very few were confirmed histologically. Those animals found dead and too autolysed for histology were only included as positive for a neoplasm if the postmortem appearance was such as to justify the diagnosis without doubt.

Histology

Liver. The criteria used in this study for the diagnosis of hepatic carcinoma are those used in previous reports (2–4) and similar to those used by Newberne and Wogan (11) describing aflatoxin-induced tumors. In the absence of metastasis both macroscopic and microscopic criteria are used; size is not a reliable guide. Popper and Schaffner (12) have suggested a minimum diameter of 1 cm, while Reuber limited hepatocellular carcinomas to 0.5 cm. The degree of anaplasia seen in the hepatocarcinomas did not correspond to the macroscopic tumor size. Histologically, hemorrhage and necrosis, local invasion, cellular pleomorphism, and loss of polarity were considered features of carcinoma.

The histologic types of hepatic tumors seen in this series will not be described in detail and were similar to those reported in previous experiments using contaminated peanut meal (3, 4) and pure aflatoxin B_1 (11). These patterns have been described by Stewart and Snell (14) as trabecular, adenomatous, and anaplastic. In this series all types were seen, frequently in mixed forms. The histologic appearance of the hepatic tumor induced by aflatoxins B_1 and G_1 in either sex was similar. Two male rats receiving 1 μg/ml aflatoxin B_1 developed adenomatous tumors of the liver with a marked fibrous stroma which was not in itself considered sarcomatous. These could be considered cholangiocarcinomas (Fig. 1). A similar tumor was seen in an experiment in which

Table 1

Treatment	Daily dose (μg)	Duration (weeks)	Initial no. of animals	Survivors at Week 10	20	30	40	50	60	70	80	90	100
Aflatoxin B_1	20	20	30	29	29	29	29	28	27	25	23	12	0
	20	10	10	10	10	10	10	10	10	9	7	4	0
Aflatoxin G_1	60	20	28	26	26	26	26	25	23	23	22	9	1
	20	20	30	29	29	29	29	29	29	26	26	17	1
	20	10	10	10	10	10	10	9	9	8	8	6	0
Aflatoxin B_2	20	10	10	10	10	10	10	10	10	10	10	8	0
Controls	No treatment		30	29	29	29	29	29	27	27	27	26	23

Survival of animals treated with aflatoxins in drinking water.

Table 2

Compound	Concentration (μg/ml)	Daily dose (μg)	Duration (weeks)	Total dose (mg)	No. and sex of animals treated	No. of animals with tumors: Liver	Kidney	No. of animals with other neoplasms
Aflatoxin B_1	1	20	20	2	15 ♂	8	2	4
					15 ♀	11	0	1
Aflatoxin B_1	1	20	10	1	10 ♂	3	0	2
Aflatoxin G_1	3	60	20	6	11 ♂	9	6	5
					15 ♀	12	0	3
Aflatoxin G_1	1	20	20	2	15 ♂	2	5	2
					15 ♀	1	0	7
Aflatoxin G_1	1	20	10	1	10 ♂	1	0	1
Aflatoxin B_2	1	20	10	1	10 ♂	0	0	2
Controls	0	0	0	0	15 ♂	0	0	6
					15 ♀	0	0	

Tumors in rats treated with aflatoxins in drinking water.

contaminated peanut meal was fed to rats (3). Two of the other animals in this group showing anaplastic hepatocarcinomas (Fig. 2) had areas which had a sarcomatous pattern as described by Stewart and Snell (14) (Fig. 3). These areas were diffuse and seen mainly at the periphery of the tumor. Both the cholangiocarcinomas and the mixed neoplasms are included in the total of hepatic neoplasms.

Cirrhosis was not seen in the nonmalignant area of the livers. The two features seen most frequently were areas of benign cystadenomas and unencapsulated hyperplastic nodules. Frequently these consist of hydropic cells (Fig. 4), as has been described in other investigations of the carcinogenic action of aflatoxin (9, 11) and considered by Newberne and Wogan (11) to be degenerating hyperplastic areas. These are also described following treatment with other carcinogens (7). Other hyperplastic nodules show varying forms from comparatively normal parenchymal cells to those with eosinophilic cytoplasm and variation in nuclear size. These lesions are not included as hepatic carcinomas. Atypical hyperplastic nodules were seen in the rats receiving aflatoxin B_2 but not frank hepatocarcinomas.

Kidneys. In the treated animals, 13 tumors arising in the kidney were seen. These varied in size from 0.4 cm to 2 cm in maximum dimension. Two were in male rats receiving 1 μg/ml aflatoxin B_1 for 20 weeks, the animals being killed after a further 71 and 72 weeks. The remainder arose in rats receiving aflatoxin G_1, 6 in the group receiving 3 μg/ml and 5 in the group receiving 1 μg/ml for 20 weeks. All were male rats. The earliest renal neoplasm was seen 54 weeks after the treatment was stopped and the last at 78 weeks. Histologically these tumors showed multiple mitotic figures with areas of necrosis and hemorrhage (Fig. 5). There was a considerable degree of cellular pleomorphism; the cells were arranged in cords frequently many cells thick. Metastases were not found, but the tumors were not encapsulated and in some instances could be seen extending between normal tubules adjacent to the tumors. Both of the animals receiving aflatoxin B_1 and which developed renal tumors did not have hepatic carcinomas. Of the 11 rats receiving aflatoxin G_1 and which developed renal tumors, 5 had hepatic carcinomas. In these cases the two types of tumor were histologically different.

Other Neoplasms. In these experiments a wide range of neoplasms were seen. Three animals developed adenocarcinomas (Fig. 6) possibly arising from the Harderian gland. These tumors were not seen in the controls and are similar to those reported in hypophysectomized rats from this laboratory fed aflatoxin-contaminated peanut meal (5) and also in rats treated with urethan as neonates (15). One early invasive squamous cell carcinoma of the esophagus was seen (Fig. 7). This type of tumor has not been seen in control rats from this colony but has been readily induced by nitrosamines (6). Three meningiomas associated with the cerebellum were found but are probably not related to the dosage with aflatoxin. The other tumors seen were 3 uterine adenocarcinomas, 3 mammary gland fibroadenomas, 4 pituitary adenomas, 4 fibrosarcomas (one of which metastasized to the lung), 3 lymphomas, 2 keratoacanthomas, and 1 testicular interstitial tumor. In the controls, 2 lymphomas, 1 pituitary adenoma, and 3 mammary fibroadenomas were seen.

DISCUSSION

From these experiments there can be no doubt that aflatoxin G_1 is carcinogenic to the rat and that its potency is of the same order of magnitude as that of B_1. Significantly more tumors developed in animals exposed to 2 mg of B_1 (19/30) than developed in animals exposed to the same amount of G_1 (3/30). In 66 rats receiving aflatoxin G_1, 11 developed renal tumors. All of these tumors arose in rats fed the aflatoxin for 20 weeks (Table 2). In previous experiments using aflatoxin-contaminated peanut meals (3, 4, 13) there has always been an unexplained incidence of renal tumors. This can now be related to the aflatoxin G_1 content of the contaminated meal.

It is uncertain whether the other tumors seen in this study are related to the treatment. Although the Harderian gland neoplasms were not seen in the controls of this experiment or in two other experiments using rats from the same colony, the incidence is such that their significance is uncertain; the significance of the esophageal tumor was also undeterminable. The wide range of other tumors seen is greater than in most control series from this colony and is similar to that reported in a feeding trial with rats derived from the same stock (4). It is, however, uncertain whether the aflatoxin increases the overall incidence of extrahepatic neoplasms.

Our results indicate that aflatoxin B_2 may be a less potent hepatic carcinogen than aflatoxin B_1, although these data are insufficient to demonstrate this conclusively.

The finding that aflatoxin G_1 is carcinogenic has implications for the many surveys that have been carried out for aflatoxin contamination of food. In most of these surveys the analytic methods used determined only aflatoxin B_1, while the content of G_1 was unknown. Examination of the aflatoxins produced when *Aspergillus flavus* was grown in culture has demonstrated that considerable amounts of aflatoxin G_1 can be produced (8). These findings led us to develop a method for determination of both aflatoxins in a single food sample (1).

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Fig. 1. Liver from male rat killed 57 weeks following administration of aflatoxin B_1, 1 μg/ml for 20 weeks. Adenocarcinoma with cholangiomatous pattern and dense fibrous stroma. H & E, X 150.

Fig. 2 Liver from female rat killed 64 weeks following administration of aflatoxin B_1, 1 μg/ml for 20 weeks. Anaplastic hepatocarcinoma. H & E, X 150.

Fig. 3. Same liver as Fig. 2 showing sarcomatous pattern at periphery of tumor. H & E, X 150.

Fig. 4. Liver from male rat killed 74 weeks following administration of aflatoxin G_1, 1 μg/ml for 20 weeks. Unencapsulated nodule of atypical parenchymal cells showing hydropic change. H & E, X 150.

Fig. 5. Kidney from male rat killed 78 weeks following administration of aflatoxin G_1, 1 μg/ml for 20 weeks. Adenocarcinoma of kidney showing necrosis and mitotic activity. H & E, X 150.

Fig. 6. Male rat killed 43 weeks following administration of aflatoxin B_1, 1 μg/ml for 20 weeks shows adenocarcinoma probably arising from Harderian gland. H & E, X 150.

Fig. 7. Esophagus from female rat killed 75 weeks following ...istration of aflatoxin G_1, 1 μg/ml for 20 weeks showing early invasive squamous cell carcinoma. H & E, X 150.