The Carcinogenic Potential of Aflatoxin or Its Metabolites in Rats from Dams Fed Aflatoxin Pre- and Postpartum

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SUMMARY

Pregnant rats were fed a diet to which groundnut meal containing aflatoxin was added. The rats were fed the diet either prior to or after parturition or for both periods. The progeny developed inflammatory, hyperplastic, and neoplastic changes in the livers that were not seen in progeny of control animals fed a diet that did not contain aflatoxin. Malignant liver neoplasms were seen in the progeny but not in the dams.

INTRODUCTION

Aflatoxin B₃ is converted in the liver of a number of species, including rats, to its hydroxylated derivative aflatoxin M₁, which is found in the blood and urine of rats and in the milk of lactating rats, sheep, and cattle (3, 5, 8, 9, 10). When mixtures of aflatoxin B₁, G₁, B₂, and G₂ are fed to lactating ewes or cows, aflatoxin M₁ is the major identifiable component in milk (2, 11). Aflatoxin M₁ is excreted in the urine of humans who ingested food contaminated with aflatoxin B₁ (6), and aflatoxin has been identified in the breast milk of humans (4). Aflatoxin M₁ has been identified in commercial milk samples (9). The acute toxicity of aflatoxin B₁ and M₁ is similar. Single doses of the compounds produce similar lesions in the livers of 1-day-old ducklings (13). Aflatoxin M₁ is a potent liver carcinogen for rainbow trout (16) and produces local tumors when injected s.c. in rats (15).

This study was undertaken to assess the carcinogenic potential of aflatoxin or its metabolites in the offspring of rats fed aflatoxin prior to and after parturition.

MATERIALS AND METHODS

Sixty pregnant Wistar rats (Woodlyn Farms, Guelph, Ontario, Canada) were fed a diet to which groundnut meal containing aflatoxin (toxic meal) was added at 25 or 50% levels. The basic diet consisted of Master fox cubes (Toronto Elevators Limited, Toronto, Canada). We prepared the diets containing aflatoxin by mixing contaminated groundnut (“Rossetti”) meal with ground fox cubes. This toxic meal has been subjected to many analyses in this and other laboratories and was found to contain 10 ppm aflatoxin B₁ and 0.2 ppm B₂; no aflatoxin G₁ or G₂ has been detected (1).

The various groups were fed the toxic meal either prior to or after parturition, or for both periods. The feeding schedule is given in Table 1. There were 10 females in each group.

To accustom the dams and maintain them on the higher protein diet, we substituted soybean meal for the toxic meal at 25 or 50% levels from Day 5 of pregnancy to weaning during the periods when they were not fed the toxic meal.

The progeny tested at weaning were grouped as follows: Group 1, 55; Group 2, 36; Group 3, 74; Group 4, 26; Group 5, 69; and Group 6, 36. Fifty male and 50 female rats derived from mothers that had been fed the cubes were used as controls. All progeny were fed the fox cubes.

The maximum amount of aflatoxin that could be fed to the dams was derived from preliminary feeding trials. The dose level was such that the highest dose (50% toxic meal) was expected to cause abortions or deaths in a small percentage of the dams in the main study. Food consumption estimates were made, but these could not be highly accurate because of spillage on the bedding that was necessary for proper maintenance of the pregnant or nursing rats. Food consumption was approximately 15 g/female/day. The average estimated total consumption of aflatoxin for the various groups is shown in Table 1.

The pregnant rats were housed in individual cages maintained in an air-conditioned room at 72—74° F. At weaning, the young rats were separated according to sex and experimental group and were housed in community cages, 8 to 10 rats/group.

When clinical features suggested an abdominal tumor, the affected animal was lightly anesthetized with ether, and we gently manipulated the abdomen in an attempt to detect the presence of neoplasms. If tumors were palpated, the animal was killed and autopsied. Animals that were sacrificed because of intercurrent disease or that died on test were autopsied unless autolysis was too advanced. The remaining animals were killed and autopsied after approximately 36 months on test. Tissues were fixed in 10% buffered neutral formalin and were processed for routine histological examination.

RESULTS

Forty dams and 208 progeny of the treated groups and 65 of the control progeny were available for evaluation. The remainder died of intercurrent disease and were not suitable for good histological assessment because of autolytic change, cannibalism, or other causes. The distribution by groups of dams and offspring examined histologically is given in Table 2.
Carcinogenic Potential of Aflatoxin Metabolites

Table 1
Feeding schedule for dams
Progeny from dams in Groups 1 and 2 received mainly placental metabolites and some milk metabolites; progeny from dams in Groups 3 and 4 received only milk metabolites; and progeny from dams in Groups 5 and 6 received placental and milk metabolites.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>% toxic meal</th>
<th>Period on toxic meal</th>
<th>No. of days on toxic meal</th>
<th>Approximate intake of aflatoxin (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>Day 10 of pregnancy to parturition</td>
<td>12</td>
<td>0.45</td>
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<tr>
<td>2</td>
<td>50</td>
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<td>3</td>
<td>25</td>
<td>1 day postpartum to 10 days postpartum</td>
<td>10</td>
<td>0.40</td>
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<tr>
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<td>50</td>
<td>1 day postpartum to 10 days postpartum</td>
<td>10</td>
<td>0.75</td>
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<td>Day 10 of pregnancy to 10 days postpartum</td>
<td>22</td>
<td>0.80</td>
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<tr>
<td>6</td>
<td>50</td>
<td>Day 10 of pregnancy to 10 days postpartum</td>
<td>22</td>
<td>1.60</td>
</tr>
</tbody>
</table>

* Ten animals per group.

Table 2
Number of dams and offspring histologically examined

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Dams without offspring</th>
<th>Dams with offspring</th>
<th>Male progeny</th>
<th>Female progeny</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>3</td>
<td>22</td>
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<td>15</td>
<td>25</td>
<td>113</td>
<td>95</td>
<td>248</td>
</tr>
<tr>
<td>Control</td>
<td>39</td>
<td>26</td>
<td>65</td>
<td>65</td>
<td>130</td>
</tr>
</tbody>
</table>

Benign and malignant tumors were seen in all groups of animals, including the dams. Most of the tissues or organs except bone and striated muscle were involved.

The liver was the only organ that frequently developed tumors and tumor-like lesions in treated dams and offspring, but not in controls. The 1st liver lesion seen in a dam (30 months old) was a biliary cyst. No malignant liver tumors were seen in the dams. The 1st liver lesion in the progeny was a biliary cyst that was seen in a 21-month-old animal, and the 1st malignant tumor seen was a cholangiocarcinoma in an animal 25 months old. The mean age of the dams examined histologically was 27.85 ± 3.1 months, and that of the progeny was 27.85 ± 4.7 months. The histological diagnosis of liver lesions is given in Table 3. Several types of hyperplastic or neoplastic conditions were observed in dams and progeny and were classified as follows.

Bile Duct Proliferation

Biliary tracts contained up to 8 well-developed and histologically normal ductules usually not surrounded by fibrous tissue.

Diffuse Fibrosis

The normal liver architecture was preserved, but each single liver cell was surrounded by a thin layer of collagen. This was not associated with degneration or necrosis.

Cystic Lesions

Biliary. These cysts were usually large enough to be seen grossly and in many instances caused considerable distortion of the gross liver appearance (Fig. 1). Microscopically, each cyst was lined by a simple columnar epithelium, histologically identical to that lining bile ducts. In many cases, groups of large and small cysts presented an adenomatous appearance (Fig. 2).

Hepatic. These cysts were variable in size but did not produce the gross liver distortion seen with the biliary cysts. These cysts appeared as spaces in the liver tissue, and no specific lining cells could be identified (Fig. 5). Many of these smaller cysts contained birefringent crystals, the nature of which has not yet been identified.

Angiomatous. Some of these cysts were large enough to be seen grossly (Fig. 3). They had a definite endothelial lining (Fig. 4), many contained an acidophilic material, and some contained blood.

Nodular Hyperplasia

This lesion consisted of several well-defined areas of distorted liver architecture, but, in them, the individual liver cells may have a greater or lesser glycogen content than do the cells in the remainder of the liver. Their nuclei tended to be slightly larger than in the rest of the liver, and the nuclear chromatin distribution tended to be irregular (Figs. 6 and 7).

The following lesions were seen only in the progeny.

Cholangiocarcinoma

The 2 cases observed were well-differentiated adenocarcinomata with a moderate number of aberrant
mitotic figures. Both cases appeared to be of multicentric origin. One case developed distant metastases. Grossly, these slightly distorted the normal liver architecture, were multiple, somewhat nodular, yellow, and stood out in striking contrast to the surrounding normal appearing parenchyma (Figs. 8 and 9).

**Hepatocellular Carcinoma**

Two cases were observed. Grossly, there was considerable nodular distortion of the liver architecture. The nodules were paler than normal, and the larger ones were often umbilicated. Metastatic nodules were observed in the spleen and lymph nodes. Both cases of hepatocellular carcinoma exhibited various degrees of differentiation. The better differentiated cells strongly resembled normal liver cells and were arranged in cords or acinar structures. Other areas consisted of large masses of anaplastic cells with no definite arrangement. The larger of such areas developed central necrosis. Aberrant mitotic figures were numerous, and a few giant cells were present. Both cases had distant metastases in which the tumor cells were generally well differentiated.

The histological diagnosis of liver lesions and the distribution of lesions in the various groups are given in Table 3. There was a variety of benign and malignant tumors of organs other than the liver in dams and offspring in all groups. Primary adenocarcinoma of the gastric fundus and small intestine, "clear"-cell carcinoma of the kidney, and adenocarcinoma of the coagulating gland, all with distant metastases, were seen in animals exposed to aflatoxin but were not seen in controls.

**DISCUSSION**

These studies indicate that aflatoxin or its metabolites reaching the embryo through the placenta or milk induce alterations that result in inflammatory, hyperplastic, and neoplastic changes in the liver. In these studies, the aflatoxin was in contaminated groundnut meal that contained at least 2 aflatoxins. Studies in which purified forms of aflatoxin B1 and intubation p.o. were used would give more precise information on dose-response relationships than can be derived from this study.

The pathological changes were first observed in the livers of animals that died or were killed at about 21 months of age. The 1st malignant tumor, a cholangiocarcinoma, was seen at 25 months. This of course does not provide information on developmental aspects of the liver anomalies. It is not known whether the cystic structures were formed at an early stage in the development of the young rats or whether they developed progressively over the life-span of the rats. It appears, in some cases at least, that there was accelerated growth of the lesions in the later stages of the rats' lives since, in several instances, the abdomens became distended after many months on test; in fact, this clinical feature was often used as a determining factor for euthanasia. Certainly, time-sequence studies would clarify this question.

All of the progeny in these studies would probably receive milk metabolites. Masri et al. (10) have shown that aflatoxin M1 is present in the milk of cows several days after the
withdrawal of aflatoxin B$_1$ from the feed. Therefore, the rats from dams fed aflatoxin prior to parturition could receive milk metabolites of aflatoxin for the 1st few days postpartum. However, Masri et al. (10) found a 6-fold decrease in the concentration of the milk metabolites within the period of 1 week, and therefore it is likely that the major effect in the progeny from dams fed aflatoxin prior to gestation was induced by placentally transferred toxins. Aflatoxin B$_1$ has been identified in the milk of cows (10) but has yet to be identified in the milk of rats.

In this study, 3 of 95 females and 1 of 113 males developed malignant liver tumors and, although the numbers are small, it would appear that the neonatal female is as susceptible as the male to the hepatotoxic effects of aflatoxin or its metabolites. However, in certain strains of rats, the male is more susceptible than the female to the hepatocarcinogenic effect of aflatoxin (4,17).

Purchase and Steyn (14) have shown that there is no difference between the rate of uptake of aflatoxin B$_1$ from the stomachs of male and female rats. There was however, more aflatoxin M$_1$ in the liver, kidney, and intestine of female rats, suggesting that a female rat is capable of metabolizing aflatoxin B$_1$ at a faster rate than do males.

In this experiment, no transitional phases between benign and malignant changes were observed. The cystic liver lesions were encountered more frequently than solid lesions, and several animals had more than 1 kind of cyst. Although many animals had degenerative lesions ranging from scattered cytoplasmic hyalinization and nuclear pycnosis of single cells to diffuse liver atrophy, no apparent association was observed between necrosis and cyst formation in the case of so-called “biliary” and “angiomatous” cysts. The cysts labeled “hepatic” may have developed from a confluence of vacuoles in liver cells. As stated above, these cysts or spaces had no demonstrable specific lining and did not contain blood as did the cysts described by Cruickshank and Sparshott (7).

The case diagnosed as cholangiocarcinoma had no accompanying biliary cysts, and all of our cases with biliary cysts had an entirely benign appearance. Papillary epithelial outgrowths as described by Cruickshank and Sparshott (7) were not seen in our series.

Newberne and Wogan (12) described 3 separate lesions which they called foci of hyperplasia, hyperplastic nodules, and hepatomata. Essentially, all 3 of these lesions were seen in our group and have been designated collectively as “nodular hyperplasia.” The International Agency for Research on Cancer (18) has recommended the abandonment of the term “hepatoma” and suggests that the term nodular hyperplasia is preferred to “regeneration nodules.” In our cases, the growth pattern was essentially disorderly, but cell atypia was not observed. Although there was a quite distinct boundary to the nodules, compression of the surrounding parenchyma was not present. As these nodules were present in the absence of inflammation, necrosis, or cirrhosis of the remainder of the liver, we would consider them to be neoplastic, but none showed malignant characteristics. The 2 cases diagnosed as hepatocellular carcinoma were not accompanied by nodular hyperplasia and were clearly malignant histologically, apart from the fact that both metastasized, one to lung and spleen and the other to lymph nodes. Despite areas of anaplasia in both livers, the metastases were very well differentiated. One of these 2 animals also had a primary adenocarcinoma of the pancreas with its own separate metastases. The 2 tumors bore no histological resemblance to each other. The offspring that developed liver cell carcinoma were exposed to the aflatoxin for the longest period (Groups 5 and 6).

There is no apparent clear-cut relationship between type and incidence of liver lesions and amount of administration of aflatoxin to the dams. No dose relationship was apparent in the large number and variety of benign and malignant tumors of other organs. The treated dams and their offspring had, however, a higher incidence of tumor production generally than did the controls in this experiment.

REFERENCES


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Fig. 1. Liver from a dam fed an aflatoxin-contaminated diet. This is typical of the gross appearance of biliary cysts.

Fig. 2. Histological section of biliary cysts, lined by simple columnar epithelium. Hematoxylin-phloxine-saffron, X 130.

Fig. 3. Liver from the progeny of a dam fed an aflatoxin-contaminated diet. Gross appearance of angiomatous cysts, appearing as dark, raised nodules (arrows) in the liver, with coexisting nodular hyperplasia.

Fig. 4. Histological section of angiomatous cysts with endothelial lining. Hematoxylin-phloxine-saffron, X 130.

Fig. 5. Histological section of hepatic cyst with irregular outline and absence of lining. Hematoxylin-phloxine-saffron, X 130.
Fig. 6. Liver from the progeny of a dam fed an aflatoxin-contaminated diet. Gross appearance of nodular hyperplasia in the absence of cirrhosis.

Fig. 7. Histological section of nodular hyperplasia (below), clearly distinct from normal liver (above). Hematoxylin-phloxine-saffron, × 130.

Fig. 8. Liver from the progeny of a dam fed an aflatoxin-contaminated diet. Gross appearance of cholangiocarcinoma in flat and raised areas of light yellow discoloration.

Fig. 9. Histological section of primary cholangiocarcinoma. Hematoxylin-phloxine-saffron, × 700.
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