The Effects of Aflatoxin B₁ and Dimethylsulfoxide on Thymidine-³H Uptake and Mitosis in Rat Liver

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SUMMARY

DNA synthesis, measured by thymidine-³H labeling of nuclei in autoradiographs, and mitosis have been studied in the livers of 148 weanling Fischer rats given a single intragastric dose of aflatoxin B₁ in dimethylsulfoxide and in 190 control rats given either dimethylsulfoxide, isotonic sodium chloride, or no treatment. A marked reduction in both DNA synthesis and mitosis in the parenchymal cells occurred within 7 hr after the administration of aflatoxin and persisted for approximately 50 hr, after which both parameters of cell division returned to normal. DNA synthesis was reduced in the Kupffer cells also, but less severely than in the parenchymal cells. There was histologic evidence of damage to both parenchymal and Kupffer cells within 3 hr after the administration of aflatoxin. Controls given dimethylsulfoxide showed an elevation of hepatic parenchymal mitoses 6 through 50 hr later in 2 out of 4 experiments and no significant change in the other 2 experiments. There was vacuolation of the liver cells but no other histologic abnormality.

INTRODUCTION

Knowledge of the histologic, ultrastructural, and biochemical alterations associated with experimental carcinogenesis, particularly in the liver, has increased rapidly in recent years. Changes in cytoplasmic, nuclear, and nucleolar structures (7, 13, 21, 27, 31, 36, 44-46, 48) and interference with the synthesis and/or function of the nucleic acids and proteins (7, 8, 10, 11, 18, 23, 29, 32, 35) have been demonstrated as acute or subacute effects of several types of carcinogens. The relation between these acute effects, which may be the result of the toxicity of the compound used, and the development of carcinoma is not known. Comparison of the response of the hepatocytes and of the other cells of the liver to a carcinogen may help to establish which of the acute changes are associated with the development of malignancy in the hepatocytes. Autoradiography can give information on certain functions of specific cells and has been used in the present study to investigate the effects of aflatoxin B₁ on the incorporation of thymidine-³H into hepatocytes and other cells of the liver.

Aflatoxin, a metabolite of the fungus Aspergillus flavus, is a hepatic toxin and a carcinogen when given in repeated doses in many species of animals (33, 34, 47). In acute studies in rats following the administration of the LD₅₀, it caused extensive necrosis in the liver followed by a delayed mitotic response (5). A single dose has been reported to decrease nucleic acid synthesis in liver regenerating after surgical partial hepatectomy (10) and to decrease the synthesis of RNA and of enzyme protein in normal liver (8, 18, 35).

We have used a single oral dose of approximately ⅓ the LD₅₀ of aflatoxin B₁, the most toxic fraction of the mold product (2, 34). This single dose, which has not been found to be carcinogenic, is approximately ⅓ of our minimum carcinogenic dose when given over a 10-day period.

MATERIALS AND METHODS

Three hundred and thirty-eight male, Fischer-strain rats, 3-5 weeks of age (A. R. Schmidt Co., Madison, Wisconsin), were used. They were maintained in an air-conditioned room at 74 ± 1°F and 50% humidity, and were fed ad libitum Purina laboratory chow. Crystalline aflatoxin B₁ (2, 34) was administered in a dose of 3 mg/kg of body weight dissolved in 0.1 or 0.5 ml of dimethylsulfoxide (Matheson, Coleman, and Bell, m.p. 18-19°C). Control rats were given 0.1 or 0.5 ml of dimethylsulfoxide alone or 0.1 ml of isotonic (0.9%) sodium chloride. All substances were given by gastric intubation between 9 and 11 A.M.

Three hr before sacrifice thymidine-methyl-³H (specific activity 3.0 c/mmole, Schwarz Biochemicals, Orangeburg, New Jersey) was given intraperitoneally in a dose of 1 μc/gm of body weight. The rats were then fasted and were sacrificed by decapitation. All rats were sacrificed between 11 A.M. and 1 P.M. except for those studied at intervals during the first 24 hr. At autopsy the left anterior lobe of the liver was frozen for lipid extraction. Sections from the median anterior and triangular lobes and from the lung, heart, kidney, pancreas, and spleen were fixed in 10% neutral buffered formalin (24). They were sectioned at 6-8 μ. Emulsion autoradiographs were prepared, using Kodak NTB₃ nuclear track emulsion (17). They were exposed for 3 weeks, developed using Kodak D19 developer, and stained with hematoxylin and eosin (25).

Counts were made in the autoradiographs of ³H-labeled hepatic parenchymal cell nuclei in 100 fields (× 450) composed of 50 consecutive fields in each of the 2 lobes taken at autopsy. ³H-labeled Kupffer cell nuclei were counted in 25 consecutive fields (× 450). The number of parenchymal cells and of Kupffer cells per field was counted, and the results were expressed as the percent of parenchymal or Kupffer cell nuclei labeled. Counts of hepatic parenchymal cells in mitosis were made in 50 fields (× 450) composed of 25 consecutive fields from each of the 2
lobes taken at autopsy, and the results were expressed as the percent of parenchymal cells in mitosis. In those cases in which thymidine-3H uptake by the bile duct cells was studied, 100 cells in well-formed ducts were examined.

The total lipid content of the liver was measured gravimetrically after extraction by chloroform:methanol (2:1) in a Soxhlet apparatus for 3 hr.

Four experiments were carried out.

Experiment 1

Thirty-five rats given 3 mg of aflatoxin B1/kg of body weight in 0.1 ml of dimethylsulfoxide and 35 rats given 0.1 ml of dimethylsulfoxide were studied 1, 3, 6, 12, and 24 hr after treatment.

Experiment 2

Fifty-two rats were given 3 mg of aflatoxin B1/kg of body weight in either 0.1 ml of dimethylsulfoxide (26 rats) or 0.5 ml of dimethylsulfoxide (26 rats). Twenty-nine rats were given 0.1 ml of dimethylsulfoxide and 30 rats were given 0.5 ml. Twenty-five rats were given 0.1 ml of 0.9% sodium chloride. They were studied 7, 26, 50, 74, and 98 hr and 1 week after treatment.

Experiment 3

Sixty-one rats given 3 mg of aflatoxin B1/kg of body weight in 0.1 ml of dimethylsulfoxide and 35 rats given 0.1 ml of dimethylsulfoxide were studied 6, 26, and 50 hr and 1, 4, 8, and 21 weeks after treatment. Four untreated rats were sacrificed with the rats studied at 26 hr. Mitoses were counted in all the rats. Thymidine-3H was given to some rats and uridine-3H to others on the same schedule and in the same dose. Therefore, the percent of mitoses is given for a greater number of rats than is the percent of 3H-labeled nuclei. The uptake of uridine-3H was not sufficient to give good autoradiographs and is not discussed further.

Experiment 4

Eleven rats given 0.1 ml of dimethylsulfoxide, 10 rats given 0.1 ml of 0.9% sodium chloride, and 10 untreated rats were sacrificed 26 or 50 hr after treatment.

The results from all experiments were analyzed by means of the t test for small samples. Differences between groups were considered significant if the P value was 5% or less.

RESULTS

Histology

There were no gross abnormalities in the liver or other organs at autopsy in any group of rats. In rats given aflatoxin B1 there was histologic evidence of liver damage 3 hr after treatment, increasing in amount through 24 hr and then decreasing until the liver appeared normal at 1 week. The most striking finding was an accumulation of basophilic debris in the Kupffer cells (Figs. 1, 2). The debris was probably remnants of nuclear material from the Kupffer cells and from damaged parenchymal cells. It was found in 86% of the livers examined 3-26 hr after the administration of aflatoxin and in 64% of the livers examined at 50 hr, and a small amount was present in 10% of the livers examined at 1 week. Most of the parenchymal cells were normal histologically, but in some there was condensation of the nuclear chromatin or hyalinized eosinophilic cytoplasm. There were occasional areas of focal necrosis (Fig. 3). Hyaline, eosinophilic bodies resembling Councilman bodies were seen frequently and appeared to be derived from the debris-filled Kupffer cells. It has been suggested that Councilman bodies in viral hepatitis are damaged Kupffer cells (41). All these changes were most marked in perportal areas but occurred in all areas of the lobule.

In portal areas at 50 through 74 hr there was an increased number of cells resembling bile duct cells, although they did not appear to be forming ducts.

In 16% of the livers of rats given dimethylsulfoxide alone and sacrificed at 3-26 hr there were a few Kupffer cells containing small amounts of debris. Councilman-like bodies were rarely seen. There was vacuolation of the cytoplasm of liver cells in some cases, as has been described previously (4, 14).

The livers studied from 1 to 21 weeks after treatment were histologically normal with no evidence of malignancy or of increased numbers of bile duct cells. There were no histologic abnormalities in the other tissues.

Cell Proliferation

The rats used were young and therefore had a high rate of cell proliferation in their livers (37, 43). The variability encountered in the indices of cell proliferation in normal rats and in rats which have undergone various experimental procedures has been discussed previously (16, 22, 28, 38, 43).

In all experiments the control group of major importance was composed of rats given the solvent dimethylsulfoxide alone. In Experiments 2, 3, and 4 data are given for additional control rats given either no treatment or 0.1 ml of 0.9% sodium chloride by gastric intubation.

Aflatoxin B1 markedly and rapidly lowered the percent of parenchymal liver cells which took up thymidine-3H or entered mitosis. In Experiment 1 the beginning of the effect on mitosis was seen at 1 hr (Chart 1). The difference between the aflatoxin-treated rats and their solvent controls was statistically significant at 12 and 24 hr after treatment. If the percent of cells in mitosis in the 2 groups studied at 15 min is considered a base line value, aflatoxin significantly lowered the percent of parenchymal cells in mitosis within 3 hr. A significant increase in hepatic mitoses over the base line value was found in rats given dimethylsulfoxide alone and studied 12 hr after treatment. The value was still elevated at 24 hr.

In Experiment 2 the results in rats given 0.1 or 0.5 ml of dimethylsulfoxide were combined, since no difference was found between the 2 groups. Parenchymal thymidine-3H uptake was significantly lowered by aflatoxin at 7 and 26 hr (Table 1). The number of grains per labeled nucleus was approximately 1/4 the number found in the solvent controls at 7, 26, and 50 hr (Figs. 4, 5). In the aflatoxin-treated rats there was an average of 31 grains/labeled nucleus with a range of 10-50. In the controls there was an average of 66 grains/nucleus with a range of 20-100. Aflatoxin-treated rats had significantly fewer mitoses than the solvent controls at 26 hr. Mitoses were still decreased at 50...
Mitosis

O.1

Mitosis

0.01

0.01

1/4 1/2 3 6 12 24

Hours after treatment

Chart 1. The acute effects of aflatoxin B₁ and dimethylsulfoxide on mitosis in rat liver parenchymal cells. Experiment 1.

hr. Dimethylsulfoxide had no significant effect on either parameter of cell division in this experiment.

In Experiment 3 the acute effects of aflatoxin B₁ and of dimethylsulfoxide on parenchymal mitosis were similar to those seen in Experiment 1. Aflatoxin-treated rats had significantly fewer mitoses than did their solvent-treated controls at 6 and 26 hr (Chart 2). The effect persisted through 50 hr. Mitoses were significantly increased over normal in dimethylsulfoxide-treated rats at 6 and 26 hr after treatment and probably at 50 hr also. Aflatoxin partially or completely prevented this increase in mitosis. Parenchymal uptake of thymidine-³H by 4 aflatoxin-treated rats was approximately 5% of the uptake in 2 solvent-treated controls at 26 hr and approximately 20% at 50 hr.

In the rats studied 1–21 weeks after treatment there were no consistent differences between the groups in the uptake of thymidine-³H or in mitosis.

In Experiment 4 the effect of intubation and the administration of sodium chloride or dimethylsulfoxide was studied. In rats given sodium chloride there was an increase in parenchymal uptake of thymidine-³H at 26 hr but no change in mitosis (Table 2). Dimethylsulfoxide had no significant effect on thymidine-³H uptake or mitosis.

The Kupffer cells varied in their response to aflatoxin (Table 3). In Experiment 2 the uptake of thymidine-³H by the Kupffer cells was significantly reduced in aflatoxin-treated rats at 26 hr. In Experiment 3 there was no effect. The administration of dimethylsulfoxide or sodium chloride in Experiment 2 was associated with an increased uptake of thymidine-³H by Kupffer cells at 50 hr. In Experiment 4 neither sodium chloride nor dimethylsulfoxide altered the percent of labeled Kupffer cells from the normal value of 1.29%.

In Experiment 2 the labeling of the nuclei of bile duct cells by thymidine-³H was examined. In the saline-treated and solvent-treated controls, 0–2% of the cells were labeled. Twenty-six hr after the administration of aflatoxin 3 of 10 rats showed increased labeling (4–8%), and at 50 hr 6 of 10 rats showed an increase (4–16%) (Fig. 6). The uptake of thymidine-³H then returned to normal. There was an increase in cells which appeared to be bile duct cells in the portal areas as well.

The total liver lipid content of rats 26 hr after treatment with aflatoxin or dimethylsulfoxide, 5.3 and 5.5% of the wet weight, respectively, was normal.

Table 1

The Effect of Aflatoxin B₁ and Dimethylsulfoxide on Mitosis in Rats

<table>
<thead>
<tr>
<th>Time between treatment and sacrifice (hr)</th>
<th>No. of rats</th>
<th>Hepatic parenchymal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO, 0.1 ml</td>
<td>AFB₁, 3 mg/kg in dimethylsulfoxide, 0.1 ml</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>26</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>74</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>98</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>1 wk.</td>
<td>9</td>
<td>5</td>
</tr>
</tbody>
</table>

Saline controls                              25 | 0.76 ± 0.19 | 0.37 ± 0.10 |

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In this study aflatoxin \( B_1 \) was found to cause a rapid and marked depression of DNA synthesis, measured by the uptake of thymidine-\(^{3}H\), and mitosis in the hepatic parenchymal cells of normal, weanling rats. DNA synthesis was affected 7 hr after administration of the compound, the earliest time at which it was studied. The decrease in mitosis began between 1 and 3 hr. The changes persisted for 26-50 hr, after which the uptake of thymidine-\(^{3}H\) and the mitotic rate returned to normal. These indices of cell division then remained normal up to 21 weeks.

As the number of \(^{3}H\)-labeled parenchymal nuclei decreased, the number of silver grains in the emulsion overlying the labeled nuclei also was reduced. This suggested that aflatoxin blocked the progression of DNA synthesis after initiation and may have blocked the initiation as well.

A rapid depression of thymidine-\(^{3}H\) incorporation into DNA has been reported in older rats when surgical partial hepatectomy was followed by the injection of a single dose of aflatoxin \( B_1 \) of approximately 1 mg/kg of body weight (10). The effect on mitoses was not described. It was stated that the percent of nuclei labeled was not reduced but that there were fewer silver grains visible in the emulsion per labeled nucleus. The majority of the labeled nuclei counted contained 3-20 silver grains. In the present study only nuclei with at least 10 or more grains were counted in order to distinguish them clearly from the background label of 0-3 grains in a similar area. This probably explains the difference in results. It is possible that the smaller dose of aflatoxin, acting on a larger number of cells being stimulated to divide following partial hepatectomy, inhibited the progression but not the initiation of DNA synthesis.

The rapid decrease in mitoses indicated that aflatoxin inhibited mitosis directly as well as indirectly through decreasing DNA synthesis. Reduction of the number of mitoses and evidence of chromosome damage have been reported in \( Vicia faba \) seedlings cultured in a medium containing aflatoxin (26). All phases
of mitosis were seen in the affected livers in the present study, so there was no evidence of an arrest of mitoses which were in progress at the time of exposure to aflatoxin.

As the liver cells recovered from the effects of aflatoxin, 3H-labeled nuclei and mitotic figures were found in all sections of the lobule. There was a normal variation between different areas and lobes, as there was between different animals, but no consistent differences in localization of cell division were found.

In biochemical studies nuclear RNA synthesis (8, 18) and the synthesis of enzyme proteins (8, 18, 35) in the liver have been shown to be decreased within the first 24 hr after administration of a single dose of aflatoxin B1. Reduction of DNA synthesis by aflatoxin has been demonstrated in vitro, using cultured embryonic lung cells (23). The binding of aflatoxin to DNA in vitro caused physical changes in the DNA (8, 42) which could lead to defects in its functioning in the synthesis of the nucleic acids and, ultimately, in the synthesis of protein.

Morphologic evidence of cell damage was present in the livers of aflatoxin-treated rats within 3 hr. The changes in the parenchymal cells were similar to but less marked than those which have been described previously following larger doses of aflatoxin (5, 33, 34, 47). Recent electron microscopic studies have described abnormalities of the rough endoplasmic reticulum and the nucleolus within 1–3 hr following a single dose of aflatoxin (6, 44–46).

The significance of these acute biochemical and morphologic changes for the cellular mechanism of aflatoxin carcinogenesis is not known. The tumors which are induced by aflatoxin are composed of malignant cells which appear to be derived from the hepatic parenchymal cells (33, 47). Therefore, aflatoxin must affect the parenchymal cells in a way which differs from its effect on the other cells of the liver. The difference may lie in a direct effect on the carcinogen or its metabolites or in the response of the parenchymal cells to a common effect. To study this, a comparison was made of the actions of aflatoxin on the parenchymal and on the Kupffer cells. There was histologic evidence of damage to both within 3 hr, which indicated that aflatoxin was toxic to both types of cell. DNA synthesis was depressed in both, although there was a difference in the severity of the depression. In Experiment 2 thymidine-3H uptake by the parenchymal cells was reduced to approximately 1/4 of the control value at 7 hr and to 1/2 of the control at 26 hr. In the Kupffer cells the reduction was to 1/2 the control value at 7 hr, which was not statistically significant, and to approximately 1/3 of the control at 26 hr. Therefore, the effect on DNA synthesis in the Kupffer cells occurred later and was less marked than the effect in the parenchymal cells. Recovery occurred at about the same time in the 2 types of cell. In Experiment 3 the histologic changes and the reduction in parenchymal thymidine-3H uptake were approximately the same as in Experiment 2, but the Kupffer cell uptake of thymidine-3H was not reduced at the times studied. Phagocytosis of 3H-labeled parenchymal nuclei and incorporation of the labeled material may have contributed to the Kupffer cell labeling, but this was probably not of importance since only 3 hr had elapsed between the injection of thymidine-3H and sacrifice. Mitoses in Kupffer cells were not counted because of difficulty in recognizing mitotic figures in the debris-laden cells.

In summary, both parenchymal and Kupffer cells were structurally damaged by the compound at about the same time. DNA synthesis was decreased in both, but the parenchymal cells were more severely and consistently affected. This apparent sensitivity of the parenchymal cells could be the result of either a difference in response to a given amount of aflatoxin or an increased uptake of aflatoxin. The morphologic and biochemical changes may have been manifestations only of the toxicity of the dose of aflatoxin. If the repeated doses required for the induction of hepatomas maintain the partially selective action on parenchymal DNA synthesis found with a single dose, then this may be a factor in the development of malignancy.

No permanent effect of the single dose of aflatoxin on cell division was found. It is possible that a stimulus such as partial hepatectomy might reveal a persisting abnormality similar to that found following X-irradiation of the liver (1) and the administration of other hepatocarcinogens (30).

The bile duct cells, which undergo a benign proliferation in rats following treatment with aflatoxin (33), responded differently from the parenchymal and Kupffer cells. There was no histologic evidence of damage to them and no decrease in thymidine-3H uptake from the normally low level. There was an increased uptake of thymidine-3H at 26 and 50 hr, and there appeared to be more bile duct cells than normal in the liver at 50 and 74 hr. By 1 week these cells had disappeared, and no further abnormality of the ducts was seen.

The inconsistent effects of the administration of dimethylsulfoxide and, to a lesser extent, of sodium chloride on cell division emphasize the importance of controls for the solvents and procedures used in a study such as this. This point has been discussed in relation to the variable effects of dietary intake (22, 28), sham operation (38) and injection of plasma (16) on thymidine-3H uptake, or other measurements of DNA synthesis and mitosis in the liver.

Dimethylsulfoxide has been widely used as a solvent for many substances in industry and medicine (4, 40), as a preservative in freezing cells (14, 49), and, until recently, as a therapeutic agent in human and veterinary medicine (9, 12, 20, 39). It has been found to be associated with the development of abnormalities of the refractive power of the lens in dogs (19). Tests for acute and chronic toxicity have also suggested the possibility of an effect of dimethylsulfoxide on the liver. In rats given repeated doses there was vacuolation of the liver cells (4, 14). Mice given dimethylsulfoxide had a prolongation of the hexobarbital sleeping time (3), which may have been the result of decreased detoxification of the barbiturate in the liver. Rhesus monkeys given intravenous infusions of dimethylsulfoxide were found to have "marked fluctuations" in the serum glutamic pyruvic transaminase activity as well as in several other laboratory tests (15). There have been no clinical reports of a toxic effect of the drug on the liver.

In the present study the administration of dimethylsulfoxide alone was associated with a rise in hepatic cell mitoses in 2 experiments. Different bottles of dimethylsulfoxide obtained from the same source were used in the various experiments. The differing results may have been due to impurity or deterioration of the compound or to a difference in sensitivity of the different groups of rats. A part of the effect may have been due to the intubation and instillation of fluid into the stomach since 1 group of rats given sodium chloride by intubation showed an increase in 3H-labeled parenchymal cells 26 hr later, although there was...
no change in mitoses. Because of the variation in the results and
the question of the role of the procedure alone, no definite state-
ment can be made on the effect of dimethylsulfoxide on cell
division in the liver. Further study of its action and the action
of the products of its degradation is indicated.

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Fig. 1. Kupffer cell containing debris (arrow) in the liver of a rat given 3 mg/kg of aflatoxin B1 7 hr before sacrifice. H & E, × 750.

Fig. 2. Debris in the sinusoids probably in Kupffer cells (arrows) in the liver of a rat given 3 mg/kg of aflatoxin B1 7 hr before sacrifice. H & E, × 500.
FIG. 3. Small area of parenchymal damage in the liver of a rat given 3 mg/kg of aflatoxin B\textsubscript{1} 26 hr before sacrifice. There are degenerated cells (black arrows), and several of the intact cells have hyalinized cytoplasm with increased eosinophilia (open arrows). H \& E, $\times$ 500.

FIG. 4. Three $^3$H-labeled parenchymal nuclei in the liver of a control rat given 0.1 ml of dimethylsulfoxide 7 hr before sacrifice. Emulsion autoradiograph, $\times$ 500.
Fig. 5. Three ³H-labeled parenchymal nuclei (arrows) in the liver of a rat given 3 mg/kg of aflatoxin B₁ 7 hr before sacrifice. The nuclei have considerably fewer overlying grains than have labeled nuclei in control rats (Fig. 4). Emulsion autoradiograph, × 500.

Fig. 6. ³H-labeled bile duct cells (arrows) in the liver of a rat given 3 mg/kg of aflatoxin B₁ 50 hr before sacrifice. Emulsion autoradiograph, × 500.
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