ABSTRACT

The influence of dietary protein content and dietary vitamin B₁₂ supplementation on the hepatotoxicity and carcinogenicity of aflatoxins in rat liver was studied. In animals fed a low-protein diet, aflatoxin induced extensive toxic and carcinogenic effects. Cirrhosis was significantly prevented to a certain level by vitamin B₁₂ administration, but the incidence of cholangiobiosis and hyperplastic nodules was unchanged. No toxic effect was observed in animals receiving high-protein diet with no vitamin B₁₂ supplement in this study (33 weeks). Only one rat bearing a hepatoma was observed in this group. However, hepatoma and hyperplastic nodules were found in the group receiving high-protein diet plus vitamin B₁₂. Cholangiobiosis and cirrhosis were not observed in the high-protein group regardless of vitamin B₁₂ administration.

INTRODUCTION

Vitamin deficiencies, malnutrition, and aflatoxins coexist in many areas of the world where there is a high incidence of liver disease including hepatocarcinoma (8, 18, 61). Aflatoxins, a group of metabolites produced by certain strains of the mold Aspergillus flavus, are presently known as potent hepatocarcinogens for rats and several other animal species (6, 15, 19, 21, 32, 33, 36, 40, 50, 62). The mold metabolites have been found as contaminants in a number of human food products and presumably constitute a public health hazard. There are numerous conflicting reports on the effects of dietary protein, deficiency in lipotrope, vitamin B₁₂, and choline on the toxicity and carcinogenicity of aflatoxins (22, 23, 31, 35, 42-45). Madhavan and Gopalan (23) found that high dietary protein could enhance aflatoxin-induced hepatoma in rat liver. Wogan and Newberne (62) reported a high incidence of hepatocarcinoma in rats continuously fed a synthetic diet high in protein and vitamin B₁₂ to which aflatoxins were added. In 1969 Rogers and Newberne (43) observed that the number of hepatic tumors found in lipotrope-deficient adult rats fed a carcinogenic dose of aflatoxin B₁ was less than the number in a control group. However, in 1971 (44) they repeated their study using weaning rats and found that the incidence of tumors was higher in the lipotrope-deficient rats. This study of the effects of dietary protein and vitamin B₁₂ on the toxicity and carcinogenicity of aflatoxins was undertaken to clarify these conflicting reports.

MATERIALS AND METHODS

We used 148 male Fischer rats (supplied by Animal Center, Faculty of Science, Mahidol University, Bangkok, Thailand), weighing 80 ± 5 (S.E.) g. They were divided into 8 groups (Table 1), housed in groups of 2 in individual cages, and fed a synthetic diet (Table 2) and water ad libitum. Daily food consumption was recorded, and the animals were weighed weekly.

Aflatoxin was obtained from Dr. G. N. Wogan (Massachusetts Institute of Technology, Cambridge, Mass.) in crystalline form. The mixture was composed of aflatoxins B₁ and G₁, approximately 1:1, and with a trace amount (about 5%) of B₂ aflatoxins and G₂. This combination of aflatoxin is regarded as a potent hepatic carcinogen (1, 19, 21, 33, 54). At the end of the experiment, the rats were killed by deep ether anesthesia. The livers were removed and weighed, and blocks were fixed in 10% buffered formalin. Paraffin sections 5 μm thick were stained routinely with hematoxylin and eosin.

RESULTS

The average growth rates of all groups are shown in Chart 1. The liver weight, the weight as a percentage of body weight, and a survey of pathological changes are shown in Table 3. Average aflatoxin consumptions (μg/rat/day) ± S.E. in each group, respectively, are: Group 3, 7.76 ± 0.63; Group 4, 7.67 ± 0.42; Group 7, 13.44 ± 0.93; Group 8, 11.60 ± 0.64.

Pathological Changes in the Liver. In the low-protein groups (Groups 1 & 2), regardless of vitamin B₁₂, the livers were small, smooth, and pale brown. Sections of these livers showed many fine vacuoles in the cytoplasm. Fatty degeneration of liver cells varied from mild to moderate. A solitary small cyst was seen in 1 liver of each group. In the vitamin B₁₂-depleted group, areas of liver cell necrosis of varying size were observed, particularly in the central area, and were replaced by fibrous tissue.

In the low-protein, aflatoxin-contaminated, vitamin B₁₂-depleted group (Group 3), the livers were small and pale reddish brown. Most of the livers showed white, pale brown, or reddish brown nodules, ranging from 0.1 to 0.5 cm in diameter, scattered throughout the surface (Fig. 1). Three of 24 rats of this group developed hepatocarcinoma. In the vitamin B₁₂-supplemented group (Group 4), the gross appearance was similar to that in the same group without vitamin B₁₂ but the nodules were smaller. Only 1 of 24 rats
developed hepatoma. Fatty degeneration, vacuolated cells, multiple cysts, perilobular fibrosis, and hyperplastic nodules were common when examined by light microscope (Figs. 2 & 3). Cholangiofibrosis (Fig. 3) and bile duct proliferation could be seen in some liver sections. Complete lobular cirrhosis invaded the lobules and distorted the normal hepatic lobular architecture. Fibrous bands of varying thickness divided the liver parenchyma into lobules corresponding with the gross nodules. These lesions were observed in Group 4 but in milder degree. The hepatic tumors observed in Groups 3 & 4 showed a pattern of trabecular hepatocarcinoma, according to the classification of Stewart and Snell (52).

In all of the high-protein groups (Groups 5 to 8), the livers...
In the present study, the high incidence of data would imply the enzymatic potentiation of the carcinogenicity of aflatoxin. In other words, the enzyme-activated metabolic product of aflatoxin possesses higher carcinogenicity than does its native form (13, 56). Nevertheless, another explanation is still debatable, since a low-protein diet may influence aflatoxin carcinogenesis in such a way that the carcinogenic process is retarded but not entirely absent. The high incidence of hyperplastic nodules and cholangiofibrosis in the protein-depleted animals is regarded as supporting evidence for the latter assumption, since these are presumably precancerous lesions (11, 27-29). If the experiment had been extended, these precancerous lesions might have progressed to frank malignancy and the ultimate result of aflatoxin-induced-neoplasms in the low- and high-protein groups may not differ.

Another lesion found to be associated with the chronic toxicity of aflatoxin was cystic formation, which was frequently seen in the protein-depleted group. Cirrhosis was absent in the high-protein group but was frequent in the animals receiving low-protein diets. This presumably was not an effect of either aflatoxin or low-protein diet alone but rather a combined effect, since the lesion was observed only in the group receiving a low-protein diet supplemented with aflatoxin. The depletion of protein could also have affected the production of detoxification enzymes (4, 5, 17, 24, 25, 48) and, as a result, the acute hepatotoxicity of aflatoxin was relatively enhanced with development of cirrhosis.

Vitamin B₁₂, one of the lipotropic factors, is also essential for the development of the hematopoietic system. Vitamin B₁₂ deficiency has been shown to induce megaloblastic anemia (2), cause subacute combined degeneration of the spinal cord in human (3), and increase the urinary excretion of N-formimino-L-glutamic acid in animals (12, 53, 59). The latter action is similar to those induced by several chemical carcinogens such as diethylnitrosamine, 2-acetylaminofluorene, and N,N-dimethyl-4-aminazobenzene. All of these carcinogens are known to interfere with enzymes involved in the metabolism of 1-carbon compounds and result in increased urinary excretion of N-formimino-L-glutamic acid (37-39). Vitamin B₁₂ supplementation had no significant effect on the reversal of this derangement. In addition, tumors associated with the liver, pancreas, and adrenals were larger than those in the low-protein groups. They were smooth and had a mahogany-colored surface. Of the 25 animals in the vitamin B₁₂-supplemented group (Group 8), 6 developed tumors (Fig. 4). Only 1 tumor was seen in the vitamin B₁₂-depleted group (Group 7). In the high-protein, vitamin B₁₂-depleted, and vitamin B₁₂-supplemented groups, most of the liver cells appeared normal except for mild fatty degeneration in the vitamin B₁₂-depleted group. In the high-protein, aflatoxin-contaminated groups (Groups 7 and 8), fatty degeneration and vacuolated cells (Fig. 5) were common lesions which present as small or large groups. In the vitamin B₁₂-depleted group, the hepatoma was of the trabecular type (52). Among 6 hepatic tumors found in the vitamin B₁₂-supplemented group, 1 was trabecular (Fig. 6), 3 were adenomatous, and 1 was mixed adenomatous and trabecular.

**DISCUSSION**

Nutritional modification of chemical carcinogenesis has long been a subject of interest (16, 34, 47, 49, 58, 60, 63). Variation in the amount of dietary protein is one of the modifications that might alter the pattern and incidence of neoplasms in the target organ, particularly the liver (23, 46, 24, 25, 48) and, as a result, the acute hepatotoxicity of aflatoxin was relatively enhanced with development of cirrhosis.

![Table 3: Liver weights and summary of histopathological changes in experimental groups](image)

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. of animals (start/finish)</th>
<th>Av. liver wt (g)</th>
<th>Av. liver wt (% body wt)</th>
<th>Cystic lesions</th>
<th>Cholangiofibrosis</th>
<th>Cirrhosis</th>
<th>Hyperplastic nodule</th>
<th>Hepatoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Low protein</td>
<td>12/6</td>
<td>5.0 ± 1.1⁹</td>
<td>5.9 ± 0.7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. Low protein + vitamin B₁₂</td>
<td>12/12</td>
<td>5.1 ± 1.4</td>
<td>5.6 ± 0.7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3. Low protein + aflatoxin</td>
<td>25/23</td>
<td>8.1 ± 1.6</td>
<td>6.5 ± 0.9</td>
<td>14</td>
<td>9</td>
<td>21</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>4. Low protein + vitamin B₁₂ + aflatoxin</td>
<td>25/24</td>
<td>8.1 ± 1.3</td>
<td>6.3 ± 0.8</td>
<td>14b</td>
<td>8b</td>
<td>12c</td>
<td>15b</td>
<td>1b</td>
</tr>
<tr>
<td>5. High protein</td>
<td>12/9</td>
<td>10.5 ± 1.6</td>
<td>3.2 ± 0.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6. High protein + vitamin B₁₂</td>
<td>12/10</td>
<td>11.8 ± 1.4</td>
<td>3.2 ± 0.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7. High protein + aflatoxin</td>
<td>25/24</td>
<td>13.9 ± 2.5</td>
<td>3.4 ± 0.4</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>8. High protein + vitamin B₁₂ + aflatoxin</td>
<td>25/25</td>
<td>13.5 ± 2.2</td>
<td>3.6 ± 0.4</td>
<td>2c</td>
<td>0</td>
<td>0</td>
<td>4c</td>
<td>6c</td>
</tr>
</tbody>
</table>

a Mean ± S.E.

b χ² test: p > 0.5 (Groups 2 and 3).

c χ² test: p < 0.05 (Groups 2 and 3, 7 and 8).

Note 1: The total liver weight was significantly greater in the vitamin B₁₂-supplemented group (Group 8) than in the vitamin B₁₂-depleted group (Group 7), and the difference was statistically significant (p < 0.05).

Note 2: The liver weight as a percentage of body weight was significantly greater in the vitamin B₁₂-supplemented group (Group 8) than in the vitamin B₁₂-depleted group (Group 7), and the difference was statistically significant (p < 0.05).

Note 3: The incidence of cystic lesions was significantly greater in the vitamin B₁₂-supplemented group (Group 8) than in the vitamin B₁₂-depleted group (Group 7), and the difference was statistically significant (p < 0.05).

Note 4: The incidence of cholangiofibrosis was significantly greater in the vitamin B₁₂-supplemented group (Group 8) than in the vitamin B₁₂-depleted group (Group 7), and the difference was statistically significant (p < 0.05).

Note 5: The incidence of cirrhosis was significantly greater in the vitamin B₁₂-supplemented group (Group 8) than in the vitamin B₁₂-depleted group (Group 7), and the difference was statistically significant (p < 0.05).

Note 6: The incidence of hyperplastic nodules was significantly greater in the vitamin B₁₂-supplemented group (Group 8) than in the vitamin B₁₂-depleted group (Group 7), and the difference was statistically significant (p < 0.05).

Note 7: The incidence of hepatomas was significantly greater in the vitamin B₁₂-supplemented group (Group 8) than in the vitamin B₁₂-depleted group (Group 7), and the difference was statistically significant (p < 0.05).

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show lower vitamin B$_{12}$ concentration than do the normal tissues (7, 20).

The influence of vitamin B$_{12}$ on aflatoxin carcinogenesis in the low-protein group is not significant in this study. Chronic dietary depletion may affect cell dynamics, particularly those with high protein turnover as in the liver and those with high cell renewal rate as in the intestinal epithelium. Consequently, the absorption of certain factors and vitamins including vitamin B$_{12}$ may be impaired (10). Hence, the influence of dietary vitamin B$_{12}$ on aflatoxin carcinogenesis in the low-protein group seemed negligible in this study.

Vitamin B$_{12}$ supplementation significantly enhanced the induction of both hyperplastic nodules and hepatoma by aflatoxin in the high-protein group. Poirier et al. reported the similar finding that vitamin B$_{12}$ could potentiate chemical carcinogenesis in rats. Day et al. (9), Georgadze (14), and Miller et al. (30) have recorded evidence of enhancement of the activity of carcinogens by vitamin B$_{12}$.

However, controversial results were observed by Rigby and Bodian (41) in 1963; this report revealed that vitamin B$_{12}$ both enhanced and inhibited tumor growth. Our observations and those of others suggest that vitamin B$_{12}$ may potentiate some chemical carcinogens in the induction of biochemical derangements and the initiation of tumor formation.

ACKNOWLEDGMENTS

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REFERENCES

Vitamin B<sub>12</sub> and Aflatoxin Carcinogenesis


Fig. 1. Typical liver from a rat fed a low-protein, aflatoxin-contaminated diet with or without vitamin B<sub>12</sub>. Hyperplastic and regenerated nodules of different sizes are observed.
Fig. 2. Typical liver from rat fed a low-protein, aflatoxin-contaminated diet, with or without vitamin B<sub>12</sub>, containing areas of cholangiofibrosis and a hyperplastic nodule. Fatty change is also seen. H & E, × 150.
Fig. 3. Liver section from the liver in Fig. 2 showing multiple cystic lesions. H & E, × 150.
Fig. 4. Liver of a rat fed a high-protein, aflatoxin-contaminated diet with or without vitamin B<sub>12</sub>. Large, yellow-brown, rounded nodules of hepatoma are seen on the surface.
Fig. 5. Liver section from a rat fed a high-protein, aflatoxin-contaminated diet with or without vitamin B<sub>12</sub>, showing foci of vacuolated cells. H & E, × 300.
Fig. 6. Liver section showing trabecular type of hepatoma from rat fed with aflatoxin-contaminated diet. H & E, × 300.
Influence of Dietary Protein and Vitamin B₁₂ on the Toxicity and Carcinogenicity of Aflatoxins in Rat Liver

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