Dietary Enhancement of Nitrosamine Carcinogenesis

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INTRODUCTION

Interactions between diet and chemical carcinogens may be responsible for geographic, socioeconomic, or other variations in occurrence of certain tumors. Cancer of the liver and esophagus is associated with cirrhosis or heavy intake of alcohol and is prevalent in certain African and Asian populations in which, as with alcoholics, malnutrition and its attendant liver dysfunction are common (16, 25, 28, 29). Malnutrition may produce both the liver disease and an enhanced susceptibility to environmental carcinogens. The importance of considering such interactions of environmental factors has recently been discussed and reemphasized (4).

Dietary deficiency of the lipotropes choline and methionine has been used extensively to produce a model in rats for human liver disease, including alcoholic fatty liver and cirrhosis, and is a useful model with which to examine dietary effects on carcinogenesis. Severely deficient rats do not tolerate carcinogen treatment well, but marginally deficient rats, which grow normally and develop fatty liver but not cirrhosis, tolerate treatment with carcinogens and live to develop tumors (20–22). When such rats were treated with AFB1,3 they developed premalignant lesions and hepatocarcinomas earlier and in greater incidence than rats fed an adequate diet (21, 22). The 2 diets differed in lipotrope and fat content and in protein source but were not deficient in protein. Before the effects of individual diets were examined, studies were undertaken to compare the effect of the 2 diets on carcinogenesis by other compounds and in other organs in order to determine whether enhancement of carcinogenesis by the deficient diet was specific for AFB1.

The 1st group of carcinogens studied was the nitrosamines, which comprise a large group of tumor-inducing compounds in many different organs of experimental animals. They are also found in human foods and are suspected to be a causative factor in human esophageal carcinoma (1, 3, 6, 8, 10, 24). The carcinogenic effect of the simplest compound, DMN, is susceptible to dietary alteration. Protein deficiency increases the number of renal tumors induced, while depressing induction of hepatic tumors, and decreases the ability of the liver to metabolize DMN (7, 13, 27).

MATERIALS AND METHODS

Nitrosamine Carcinogenesis. Four-week-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) were fed either an adequate diet (Diet 1) or a diet high in fat and marginally deficient in lipotropes (Diet 2) throughout the experiment (21). After 3 weeks they were placed on 1 of 4 regimens in groups of 25 to 34 per diet: Regimen 1, AFB1, 40 ppm in the diet; Regimen 2, DBN, 200 mg/kg body weight s.c. once a week for 15 weeks, and then 4 additional doses at intervals of 1 to 4 weeks, depending on the weight and clinical appearance of the group; Regimen 3, DMN, 100 ppm in diet for 3 weeks; Regimen 4, DMN, 25 ppm in the diet for 4 weeks followed by 50 ppm in the diet for 8 weeks. Nitrosamines were purchased from Eastman Kodak Co., Rochester, N. Y. Rats were housed singly in stainless steel, suspended cages at 70 ± 2°F and given tap water. They were weighed weekly, and food intake was measured for 1 week/month when carcinogen was fed. Rats were observed until they became moribund, at which time they were killed and autopsied. When only 3 to 5 rats remained in a group, they were all killed. The number of rats shown in the tables is the number that survived treatment.

Tissues were fixed in 10% neutral buffered formalin, processed for histology, and stained with hematoxylin and eosin.

In Vitro Hepatic Metabolism of DMN. Four-week-old male Sprague-Dawley rats were housed as described and fed Diet 1 or Diet 2 for 3 weeks, at which time hepatic DMN metabolism was measured in liver slices.

The liver was placed in ice-cold 0.9% NaCl solution and cut with a Stadie-Riggs tissue slicer into 200-mg slices, 0.5 mm thick. They were transferred to 50-ml Erlenmeyer flasks which

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3 The abbreviations used are: AFB1, aflatoxin B1; DMN, N-nitrosodimethylamine; DBN, N-nitrosodibutylamine.

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contained 2.7 ml ice-cold Krebs-Ringer phosphate buffer and had center wells, which contained 0.2 ml of 10% NaOH (12, 19). The flasks were oxygenated and capped, and DMN-14C (0.8 μCi; specific activity, 2 mCi/m mole; New England Nuclear, Boston, Mass.) was injected. After incubation for 4 hr at 37°C in a shaking water bath, the contents of the center well and Na2CO3 carrier were precipitated with BaCl2, washed with water and acetone, suspended in scintillation solvent (Cab-o-Sil, Tracerlab, Inc., Waltham, Mass.), and counted in a Nuclear Chicago Unilux II scintillation counter to give the percentage of conversion of DMN-14C to 14CO2 (9). Hepatic lipid was extracted at room temperature in methanol:chloroform (1:2) and weighed (20).

RESULTS

Rats fed N-nitrosodiethylamine or DMN in either diet grew well and appeared healthy throughout the feeding period. DMN, 100 ppm, caused decreased weight gain during the feeding period, but the rats recovered rapidly after it was removed from the diet. Toxicity of DBN was manifested by decreased weight gain after 4 injections in rats fed Diet 2 and after 8 injections in rats fed Diet 1. They had poor coats and were occasionally jaundiced. Intake of N-nitrosodiethylamine and DMN in the diet was essentially the same in the 2 diet groups (Tables 1 and 2).

N-nitrosodiethylamine. Rats fed Diet 2 died with hepatocarcinoma earlier than did rats fed Diet 1 (Chart 1). The life-span of tumor-bearing rats fed Diet 2 was 204 ± 7 (S.E.) days after initiation of N-nitrosodiethylamine treatment; in rats fed Diet 1, it was 234 ± 10 days (p < 0.02). One-half of the rats fed Diet 2 were dead with tumor at the time the 1st Diet 1-fed rat died. Esophageal tumor development probably was enhanced in rats fed Diet 2, but the difference was less striking. The probability of death with esophageal tumor rose earlier and more rapidly in rats fed Diet 2 (Chart 2) (see Ref. 23 for calculation). The esophageal tumors in most cases were small squamous polyps and were an incidental finding in rats dying of hepatocarcinoma. Tumor incidence at autopsy is given in Table 1. The incidence of multiple primary tumors was nearly doubled in rats fed Diet 2. The incidence of tumors of lung and bladder is too low to be of significance in this number of rats; however, the only malignant pulmonary tumors were found in rats fed Diet 2. The histological patterns of tumors were not otherwise different in the 2 groups, and a high percentage of hepatocarcinomas metastasized to lung.

Table 1

<table>
<thead>
<tr>
<th>Diet</th>
<th>No. of rats</th>
<th>DMN intake (total mg/rat)</th>
<th>Body wt (g)</th>
<th>% of rats with tumor in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Eosphagus</td>
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<tr>
<td>1</td>
<td>23</td>
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<td>2</td>
<td>34</td>
<td>176</td>
<td>702</td>
<td>44b</td>
</tr>
</tbody>
</table>

a Average weight at end of N-nitrosodiethylamine treatment.
b Diet 1, 13% squamous carcinoma, 22% squamous polyp; Diet 2, 9% squamous carcinoma, 35% squamous polyp.
c Hepatocarcinoma in all; in 1 case in rats fed Diet 1 and in 2 cases in rats fed Diet 2. There was also cholangiocarcinoma. In rats fed Diet 1 there was 1 case of hepatic hemangiopericytoma, which raises the percentage of liver tumors to 74%.
d Diet 1, bronchial papilloma; Diet 2, squamous carcinoma, papillary adenocarcinoma, peripheral adenoma in 1 rat each.
e Two small transitional cell polyps were found in 1 rat.

Table 2

<table>
<thead>
<tr>
<th>Diet</th>
<th>No. of rats</th>
<th>DMN intake (total mg/rat)</th>
<th>Body wt (g)</th>
<th>% of rats with tumor in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>Hepatocarcinoma</td>
</tr>
<tr>
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<td>53</td>
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</tr>
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<td>55</td>
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<tr>
<td>2</td>
<td>24</td>
<td>116</td>
<td>511</td>
<td>46</td>
</tr>
</tbody>
</table>

a Average weight at end of DMN treatment.
b In rats fed 100 ppm DMN there were single cases of pituitary and islet cell adenoma (Diet 1) and transitional cell polyps of bladder (Diet 2) in addition to the tumors listed.
c Kupffer cell sarcoma or hemangiopericytoma.
d Diet 1, 100 ppm, 12% papillary adenocarcinoma, 4% tubular adenoma; Diet 2, 100 ppm, papillary adenocarcinoma; Diet 1, 50 ppm, tubular adenoma.
e Diet 2, 100 ppm, renal hemangiopericytoma, reticulum cell sarcoma, and carcinoma of thymus, 3.3% each; Diet 1, 50 ppm, lymphoma; Diet 2, 50 ppm, abdominal fibrosarcoma.
mesentery, and/or other abdominal sites in both groups (69% in rats fed Diet 1 and 60% in rats fed Diet 2).

DBN. DBN was toxic to rats in both dietary groups, and the frequency of administration was reduced after 15 weeks. Two of 25 rats in each group died after 15 to 17 doses; 1 of each had a bladder polyp at that time. Rats were killed when they developed 2 to 3+ hematuria on testing with Labstix (Ames Co., Elkhart, Ind.) or dyspnea or when they appeared moribund. There was no difference in average life-span in the 2 dietary groups, which was 217 days from the 1st dose of DBN in rats fed Diet 1 and 214 days in rats fed Diet 2, nor in the incidence of bladder or lung tumors (Table 3). However, as with N-nitrosodiethylamine, hepatocarcinoma was found at autopsy with significantly greater frequency in rats fed Diet 2. The incidence was nearly tripled ($p < 0.05$). In contrast to the findings with $N$-nitrosodiethylamine, esophageal tumors were found only in rats fed Diet 1 as was the only malignant renal tumor.

DMN. Rats fed DMN lived longer than the other groups and had a lower incidence of tumors. Those fed 100 ppm for 3 weeks lived an average of 365 days (Diet 1) and 362 days (Diet 2) after DMN feeding was begun. Rats fed 50 ppm for 12 weeks lived 301 ± 16 days (Diet 1) and 333 ± 18 days (Diet 2). The difference is not statistically significant but is in a direction opposite to that found with $N$-nitrosodiethylamine. There were more renal tumors in rats fed 100 ppm and Diet 1 and more hepatocarcinomas in rats fed 50 ppm and Diet 1 than in rats fed Diet 2, but again the differences are not significant (Table 2). DMN induced Kupffer cell sarcomas in a few rats. Abnormalities of Kupffer cells, cystic dilation of sinusoids, and hemorrhagic hepatic cysts were found frequently in non-tumor-bearing animals.

DMN Metabolism. In rats fed Diet 1, the total hepatic lipid content was 8.0 ± 0.8%, and in rats fed Diet 2 there was a small but significant increase to 10.2 ± 0.4%. DMN$^{14}$C metabolism, measured by $^{14}$CO$_2$ production per 0.2 g dry, fat-free liver, was the same in livers from rats fed Diet 1 or Diet 2: 24.1 ± 3.9 and 21.1 ± 2.0%, respectively.

**DISCUSSION**

Enhancement of chemical carcinogenesis in the liver by feeding a high-fat, marginally lipotrope-deficient diet, first demonstrated with AFB$_1$, has been found with 2 of 3 nitrosamines tested in the present study, $N$-nitrosodiethylamine and DBN, but not with DMN. Rats fed Diet 2 and $N$-nitrosodiethylamine had increased tumors in several organs,

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**Table 3**

<table>
<thead>
<tr>
<th>Diet</th>
<th>No. of rats</th>
<th>Body wt (g)$^a$</th>
<th>% of rats with tumor in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Esophagus</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>636</td>
<td>12$^b$</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>629</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ Average weight at end of DBN treatment.

$^b$ Squamous carcinoma, 4%; squamous polyp, 8%.

$^c$ Hepatocarcinoma in all cases; difference statistically significant, $p < 0.05$.

$^d$ Diet 1, 16% squamous carcinoma, 12% transitional carcinoma, 4% adenocarcinoma, 48% transitional or squamous polyp; Diet 2, 12% squamous carcinoma, 4% transitional carcinoma, 4% adenocarcinoma, 52% transitional or squamous polyp.

$^e$ Diet 1, 88% papillary adenocarcinoma, one-half of which contained areas of squamous carcinoma, 8% squamous carcinoma, 4% adenoma; Diet 2, 76% papillary adenocarcinoma, one-half of which contained areas of squamous carcinoma, 12% adenoma.

$^f$ Diet 1, squamous carcinoma of pelvis; Diet 2, tubular adenoma.
but with DBN carcinogenesis outside the liver probably was increased in rats fed Diet 1. The disparate effects in other organs may be the result of route of administration, since DBN was given s.c. in order to induce tumors in organs other than liver (5). The incidence of tumors in DBN-treated rats is similar to that reported by Druckrey et al. (5) in BD rats, except that he found no primary lung tumors. C57Bl mice given far higher doses of DBN in drinking water developed fewer bladder tumors, more esophageal tumors, and no hepatic tumors (2).

Nucleic acid alkylation, either directly or via methionine, occurs with these higher analogs of DMN as well as with DMN itself and may be a step in carcinogenesis (11, 26). \( \text{N} \)-Nitrosodiethylamine depresses the activity of several enzymes involved in methionine and 1-carbon metabolism and decreases hepatic folate stores in rats (17, 18). If this metabolic interference is essential to its carcinogenic activity, a synergistic or additive effect with lipotrope deficiency is to be expected.

DMN yielded essentially the same results in rats in the 2 dietary groups. The toxic effects of DMN and \( \text{N} \)-nitrosodiethylamine can be dissociated by the administration of cysteine. Cysteine, 0.3 g/day s.c., significantly decreased the toxicity of DMN but not of \( \text{N} \)-nitrosodiethylamine (14, 15). Rats fed Diet 2 were taking in approximately 0.2 g of cystine per day, 5 times the amount eaten by rats fed Diet 1, and may have been protected by it against DMN. No effect of diet on DMN metabolism in vitro was found, although we have found depression of other hepatic drug-metabolizing enzymes (21). The metabolic effects underlying the observations made in the present study are far from clear, but the potential usefulness of dietary treatment to elucidate them and to alter effects of chemical carcinogens has been demonstrated. There was significant alteration of chemical carcinogenesis in rats by feeding a diet that caused either no depression of growth or, at most, a transitory depression, and there was no histological abnormality of tissues except for deposition of a small amount of fat in the liver. This suggests that in the search for causes of variations in tumor incidence in human populations one must look for not only gross dietary abnormalities but also marginal deficiencies or abnormalities that may cause only subtle metabolic changes.

REFERENCES

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