Reduction of N-Nitrosodiethylamine Carcinogenesis in Rats by Lipotrope or Amino Acid Supplementation of a Marginally Deficient Diet

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SUMMARY

In studies in this and other laboratories, induction of hepatocarcinoma by several different chemical carcinogens was enhanced in rats fed diets deficient in lipotropes (choline, methionine, folic acid), amino acids, and niacin, and high in fat. In some cases, specific supplementation with lipotropes blocked carcinogenesis.

In studies reported here, specific supplementation of a marginally deficient diet that enhanced carcinogenesis in rats, with the amino acids or lipotropes in which it was deficient, significantly decreased induction of hepatocarcinoma by N-nitrosodiethylamine. Niacin supplementation decreased hepatocarcinoma incidence only slightly; the addition of beef fat to an adequate diet did not enhance tumor induction. Rats fed the amino acid- or lipotrope-supplemented diets had an increased incidence of hepatic hemangioendothelial sarcomas, compared to deficient rats or to rats fed the adequate control diet. Methionine was contained in both the amino acid and the lipotrope supplement and probably was responsible for reducing hepatocarcinoma incidence.

Methionine has been found to have an anticarcinogenic effect in other studies and also to block the depletion of hepatic folate stores that is induced by N-nitrosodiethylamine. Interactions between carcinogens, S-adenosylmethionine, and folate may be significant in hepatic or other tissue carcinogenesis.

One or more hepatic microsomal oxidases were depressed in rats fed any of the high-fat diets but were not correlated with tumor incidence.

INTRODUCTION

Epidemiological studies give increasing evidence of environmental influences on the induction and development of cancer; the geographic and cultural distribution of cancers of particular organ sites indicate that diet is one of the major environmental factors to be considered (15). Foods may be contaminated by chemical carcinogens, e.g., aflatoxins, nitrosamines, or polycyclic aromatic hydrocarbons, or the intake of nutrients themselves may influence the incidence of certain cancers. Nutrients that have been evaluated and found to have some correlation with cancer incidence are fat, protein, vitamin A, and, possibly, folic acid and certain minerals, in particular iron, zinc, and selenium.

Studies in experimental animals support some of the epidemiological observations. Diets severely deficient in protein decreased the carcinogenicity of DMNA and AFB, for the liver but may have enhanced the carcinogenicity of DMNA for the kidney in rats (6, 8). Dietary protein altered the incidence and distribution of spontaneous tumors in rats (25). Dietary fat was positively correlated with the incidence of mammary tumors induced in rats by 7,12-dimethylbenzanthracene (2). Colon tumor induction by 1,2-dimethylhydrazine was enhanced by quadrupling but not by doubling dietary fat content (18, 22). Marginal deficiency of vitamin A enhanced induction of colon tumors by AFB (12).

Deficiency of the lipotropic factors, choline, methionine and folic acid, in high-fat diets that contained proteins deficient in certain amino acids enhanced carcinogenesis in rats treated with several different carcinogens (19, 21, 23, 28). However, excessive vitamin B12, another lipotrope, also enhanced chemical carcinogenesis (3, 11, 16). In lipotrope-deficient rats fed peanut meal contaminated with aflatoxins, choline supplementation decreased the incidence of hepatocarcinoma (28). In rats fed soy protein, which is deficient in methionine, supplementation with choline and methionine together decreased tumor induction by AFB (13). In subsequent studies in rats fed a lipotrope-deficient diet supplemented with choline to produce marginal deficiency, the carcinogenicity of AFB1, DENA, N-nitrosodibutylamine, and AAF for the liver and 1,2-dimethylhydrazine for the colon was increased compared to rats fed a diet adequate in all respects known for the rat (19, 22-24). The marginally deficient diet differed from the adequate diet not only in lipotropes but also in certain amino acids other than methionine and in fat and niacin. It supported growth well after a lag during the initial rapid growth phase following weaning; it increased hepatic cell turnover, decreased resting levels of hepatic microsomal oxidases, and blocked their induction by AFB and AAF and decreased hepatic S-adenosylmethionine (21).

The experiments reported here were undertaken to examine...
ine the effect of supplementation with the nutrients listed on enhancement of DENA hepatocarcinogenesis by the marginally deficient diet and the relationship, if any, between dietary effects on hepatic microsomal oxidases and DENA carcinogenesis.

MATERIALS AND METHODS

Acute Experiments*. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.) were fed Diets 1 to 6 ad libitum (Table 1) from weaning to 10 weeks of age. There were 10 rats/diet group; they were housed individually in stainless steel cages in air-conditioned animal quarters and weighed weekly. At the end of the feeding period, they were killed by decapitation. Hepatic lipid and microsomal oxidases, p-nitroanisole demethylase, aminopyrine demethylase, and benzo(a)pyrene hydroxylase were measured in 4 rats/group (9, 21). In 4 rats fed Diet 1 or Diet 2, hepatic NAD-NADH was measured (5).

Diet 1 (adequate) and Diet 2 (marginally deficient in lipotropes, amino acids, and niacin and high in fat) were the diets fed in the experiments cited in the “Introduction.” Proteins used in the 2 diets were analyzed for amino acid content by the laboratories of the Wisconsin Alumni Research Foundation, Madison, Wis.; the results, compared to the National Academy of Sciences/National Research Council listing of the amino acid requirements of rats, are given in Table 2 (14). Diet 2 contained inadequate amounts of methionine, arginine, glutamate, isoleucine, lysine, threonine, and tryptophan. The requirement of rats for niacin is approximately 20 mg/kg diet if tryptophan is present in adequate quantities. Therefore, the level of 12 mg/kg in Diet 2 associated with the low level of tryptophan was at least marginally deficient (14).

The other experimental diets were designed as follows: Diet 2A was Diet 2 supplemented with choline to make it equivalent in total lipotropic content to Diet 1 on a caloric basis. Diet 3 was Diet 1 altered by the addition of beef fat and corn oil in amounts equal to Diet 2 at the expense of Wesson oil and carbohydrate. Diet 4 was Diet 2 except for substitution of the vitamin mix from Diet 1, which increased the niacin content to 50 mg/kg but did not significantly alter the other vitamins. Diet 5 was Diet 2 supplemented with choline, methionine, and folic acid equal to Diet 1 on a caloric basis. (Diet 1 contained 450 kcal/100 g, and Diet 2 contained 531 kcal/100 g.) The deficient amino acids in Diet 2 were increased in Diet 6 to equal the levels in Diet 1 on a caloric basis.

Twelve male Sprague-Dawley rats fed Diet 1 and 14 rats fed Diet 2 for 3 weeks from weaning were then fed DENA (Eastman Kodak Co.; Rochester, N.Y.), 40 ppm, in the diet. Controls were fed the diets only. After 5, 11, or 17 weeks of DENA treatment, rats were exsanguinated by cardiac puncture under ether and autopsied. Serum was frozen for assay of α1-fetoprotein (kindly performed by Dr. Stewart Sell, University of California, San Diego Medical School). Hepatic lipid was extracted and measured gravimetrically.

RESULTS

Acute Experiments. Rats fed Diet 2 grew at the slowest rate; Diets 4 and 5 supported growth at a rate nearly equal to that supported by Diet 1, and rats fed Diets 3 or 6 grew most rapidly. Hepatic lipid was slightly but not significantly greater in rats fed Diets 2 to 5 than in rats fed Diets 1 or 6. One or more of the microsomal oxidases was decreased, compared to rats fed Diet 1, in rats fed any of the remaining diets, but rats fed Diets 3 and 6 had the most nearly normal levels (Table 3). In rats fed Diet 1, the hepatic content of NAD-NADH was 0.59 ± 0.07 μmole/g (± S.E.); in rats fed Diet 2, the content was 0.34 ± 0.07 (p < 0.05).

In the 2nd group of rats, serum content of α1-fetoprotein was increased in rats fed Diet 2 with no added DENA and was further increased by the addition of DENA. DENA induced a small rise at 5 and 11 weeks in rats fed Diet 1; in both diet groups, α1-fetoprotein was at basal levels after 17 weeks. After 5 weeks of DENA treatment, rats fed Diet 2 had significantly greater hepatic lipid content than their diet.

In both experiments, sections of liver and kidney were fixed in 10% neutral buffered formalin and processed by routine methods for histological examination.

Carcinogenesis Experiments. Twenty-five to 28 male, weanling, Sprague-Dawley rats were fed Diets 1, 2, or 2A (Table 1). After 3 weeks, DENA 40 ppm, was incorporated into the diets and fed for 12 weeks. Diet intake was measured 1 week of every 4 during DENA feeding. Five control rats per group were fed the diet only. Rats were weighed weekly and killed when they exhibited sustained weight loss or other evidence of hepatic tumor.

Male, weanling, Sprague-Dawley rats were fed one of Diets 1 to 6 (Table 1); there were 30 rats/diet. After 3 weeks, DENA was incorporated into the diet at 40 ppm and fed for 12 weeks. The rats were weighed weekly and killed when they exhibited signs of hepatic tumor. Food intake was measured 1 week of every 4 while DENA was fed.

At autopsy in both experiments, after examination of all organs for tumors, the major organs were fixed in 10% neutral buffered formalin, processed by routine histological methods, and examined in sections stained with hematoxylin and eosin.

Cumulative probability of death with tumor at each week was calculated (26). The calculation was made by the equation:

\[ P_x = 1 - \prod \left( \frac{(N_1 - t_i)}{N_1} \times \frac{(N_2 - t_2)}{N_2} \times \ldots \times \frac{(N_x - t_x)}{N_x} \right) \]

\[ N \] is the number of animals alive at the beginning of the week, \( t \) is the number that died with tumor during the week, and \( p \) is the probability of death with tumor. The calculation gives a more accurate assessment of alteration of carcinogenesis than is given by cumulative mortality with tumor because it takes into account death from other causes which results in a changing population at risk (26). Twenty-three to 29 of the 30 rats in each group were alive at the time the first rat died with tumor. Tumor incidences were compared statistically by \( \chi^2 \).

* The contributions of 2 undergraduate students, Jon Warren (Massachusetts Institute of Technology) and Thomasina Jedrzynski (Simmons College), to performance of this section of the study are gratefully acknowledged.
controls or rats fed Diet 1; 2 of 4 rats fed Diet 2 had focal renal cortical necrosis characteristic of acute lipotrope deficiency.

Carcinogenesis Experiments. Rats fed Diet 2A (Diet 2 + choline) had normal hepatic lipid and gained weight more rapidly than rats fed Diet 2 but not as rapidly as rats fed Diet 1.

Tumor incidence and cumulative probability of death in animals fed either Diet 2 or 2A compared to animals fed Diet 1 (Table 4). Neither the cumulative probability of death with hepatic tumor nor the final incidence of hepatic tumor was influenced by supplementation of Diet 2 with choline.

The dietary effects on weight gain were approximately the same as in the acute experiment: rats fed Diets 3 and 6 gained at the most rapid rate, and rats fed Diet 2 gained least rapidly (Table 5). Total intake of DENA varied somewhat between the groups, but there were no marked differ-

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### Table 1
Composition of experimental diets fed to male Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Component</th>
<th>1</th>
<th>2, 2A, 4</th>
<th>3</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin-free casein</td>
<td>22</td>
<td>3</td>
<td>22</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Methanol-extracted peanut</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Gelatin</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood fibrin</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose, dextrose, dextrin</td>
<td>55.7</td>
<td>36.3</td>
<td>38.7</td>
<td>35.15</td>
<td>29.6</td>
</tr>
<tr>
<td>Wesson oil</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mazola oil</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef tallow</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Amino acids*</td>
<td>5.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folic acid*</td>
<td>(0.001)</td>
<td>(0.001)</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.3</td>
<td>0.2</td>
<td>0.3</td>
<td>0.35</td>
<td>0.2</td>
</tr>
<tr>
<td>Alphacel</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* All diets were mixed into 3% agar and contained 5% Rogers-Harper's salts and 2% vitamin mix. The vitamin content of Diets 1 and 3 was, in mg/kg: vitamin A, 10; D, (IU), 3000; E, 169; menadione, 1; niacin, 50; calcium pantothenate, 20; riboflavin, 4; thiamine HCl, 8; pyridoxine HCl, 8; folic acid, 10; inositol, 250; B12, 0.05. The vitamin content of Diets 2, 5, and 6 was, in mg/kg: vitamin A, 10; D, (IU), 3000; E, 225; menadione, 10; niacin, 12; calcium pantothenate, 40; riboflavin, 16; thiamine HCl, 16; pyridoxine HCl, 16; inositol, 250; biotin, 0.07; B12, 0.05. The vitamin content of Diet 4 was the same as Diets 1 and 3 except for omission of folic acid. When DENA was fed, it was added at 40 ppm.

† Diet 2A differed from Diet 2 in choline content: 0.34% choline chloride was added at the expense of sucrose to give a total of 0.54%.

‡ Diet 4 differed from Diet 2 in vitamin content, see footnote a.

In Diets 1 and 3, carbohydrate is mixed: 36% each of dextrose and dextrin and 28% sucrose; in Diets 2, 4, and 6, carbohydrate is sucrose.

§ Arginine, 0.66 g/kg; glutamic acid, 2.45; isoleucine, 0.64; lysine, 0.98; threonine, 0.36; tryptophan, 0.15.

Diets 1 and 3 incorporated 0.001% folic acid with the vitamin mix.

### Table 2
Deficient amino acids in Diet 2 compared to level in Diet 1 and to dietary requirement of rats

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Diet 1</th>
<th>Diet 2</th>
<th>Dietary re-requirement of rats (14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>0.67</td>
<td>0.12</td>
<td>0.67</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4.22</td>
<td>2.49</td>
<td>4.40</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.97</td>
<td>0.49</td>
<td>0.61</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.43</td>
<td>0.69</td>
<td>1.00</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.55</td>
<td>0.16</td>
<td>0.67</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.68</td>
<td>0.44</td>
<td>0.56</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.22</td>
<td>0.11</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* Up to 50% of requirement can be supplied by cystine which is present at 0.10% in Diet 1 and 1.17% in Diet 2 (0.5% added + 0.67% present in protein mix).

### Table 3
Hepatic microsomal oxidases in male Sprague-Dawley rats fed experimental diets 1 to 6 from weaning to 10 weeks of age

<table>
<thead>
<tr>
<th>Diet</th>
<th>PNA*</th>
<th>BPOH</th>
<th>AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>257 ± 36</td>
<td>14 ± 4</td>
<td>73 ± 6</td>
</tr>
<tr>
<td>2</td>
<td>104 ± 6</td>
<td>9 ± 2</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>3</td>
<td>197 ± 14</td>
<td>12 ± 4</td>
<td>47 ± 10</td>
</tr>
<tr>
<td>4</td>
<td>154 ± 28</td>
<td>8 ± 3</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>5</td>
<td>111 ± 16</td>
<td>8 ± 1</td>
<td>30 ± 9</td>
</tr>
<tr>
<td>6</td>
<td>138 ± 21</td>
<td>10 ± 3</td>
<td>55 ± 19</td>
</tr>
</tbody>
</table>

* PNA, p-nitroanisole demethylase (μg p-nitrophenol/g liver/hr); BPOH, benzo(a)pyrene hydroxylase [quinine units (9)]; AP, amino- pyrine demethylase (μg aminopyrine/g liver/hr).

† Significantly less than Diet 1, p < 0.05.

Mean ± S.E.
Table 4

Incidence of hepatic and esophageal tumors in male, Sprague-Dawley rats fed Diets 1, 2, or 2A which contained DENA, 40 ppm, for 12 weeks

<table>
<thead>
<tr>
<th>Diet</th>
<th>Total DENA intake (mg/kg body wt)</th>
<th>% rats* with hepatic tumor</th>
<th>% rats* with esophageal tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Carcinoma</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>1</td>
<td>148</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>159</td>
<td>60</td>
<td>8</td>
</tr>
<tr>
<td>2A (2 + choline)</td>
<td>162</td>
<td>68</td>
<td>5</td>
</tr>
</tbody>
</table>

* Rats alive when 1st death with hepatic tumor occurred: Diet 1, 25; Diet 2, 25; Diet 2A, 22.
* Rats alive when 1st death with esophageal tumor occurred: Diet 1, 12; Diet 2, 10; Diet 2A, 8.
* Difference from Diet 1 significant, p < 0.05.

Table 5

Body weight, total intake of DENA, and hepatic tumor incidence in male, Sprague-Dawley rats fed DENA, 40 ppm for 12 weeks, in experimental Diets 1 to 6

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body wt (g) at age</th>
<th>Total DENA intake (mg/kg body wt)</th>
<th>% rats* with hepatic tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 wk</td>
<td>15 wk</td>
<td>25 wk</td>
</tr>
<tr>
<td>1</td>
<td>156</td>
<td>459</td>
<td>532</td>
</tr>
<tr>
<td>2</td>
<td>114</td>
<td>442</td>
<td>541</td>
</tr>
<tr>
<td>3 (1 + beef fat)</td>
<td>131</td>
<td>484</td>
<td>572</td>
</tr>
<tr>
<td>4 (2 + niacin)</td>
<td>110</td>
<td>444</td>
<td>547</td>
</tr>
<tr>
<td>5 (2 + lipotropes)</td>
<td>110</td>
<td>450</td>
<td>544</td>
</tr>
<tr>
<td>6 (2 + amino acids)</td>
<td>120</td>
<td>452</td>
<td>556</td>
</tr>
</tbody>
</table>

* Rats alive when 1st death with hepatic tumor occurred: Diet 1, 23; Diet 2, 28; Diet 3, 29; Diet 4, 26; Diet 5, 26; Diet 6, 24.
* Significantly different from Diet 2, p < 0.001.
* Significantly different from Diet 2, p < 0.02.
* Significantly different from Diet 3, p < 0.05.

DISCUSSION

These experiments illustrate the complexities of dietary interactions with chemical carcinogenesis. Earlier studies demonstrated that Diet 2, which was high in fat and marginally deficient in lipotropes, several amino acids, and niacin, enhanced the induction of tumors in the liver and colon; this study confirmed that result. The diet, despite its multiple deficiencies, supported weight gain and normal tissue histology except for a small amount of fat in the liver cells.
Hepatic microsomal oxidases and S-adenosylmethylenthein were significantly decreased; these alterations which were postulated to contribute to enhanced carcinogenicity of chemicals in deficient rats (1, 17, 22). In this study, there was not a consistent relationship between the hepatic oxidases measured and tumor incidence. The enzymes were reduced in all groups fed high-fat diets. Turnover of liver cells was increased in rats fed Diet 2, and this probably accounts for the small elevation of α1-fetoprotein found in this study.

Attention was focused initially on lipotropes and lipotrope supplementation because studies had demonstrated protection by choline and methionine against AFB1, AAF, and ethionine carcinogenesis (4, 10, 13, 27, 28). There was evidence also of acute interactions between lipotropes and DENA. Interference by DENA with folate metabolism was blocked by methionine or choline (17). In the acute studies presented here, DENA induced renal lesions of acute lipotrope deficiency in rats fed Diet 2 but not in rats fed Diet 1.

In all 3 studies in rats treated with DENA, it has been found to be a more effective hepatocarcinogen in rats fed Diet 2 than in rats fed Diet 1. Supplementation of Diet 2 with choline alone had no effect on tumor induction; supplementation with amino acids which included methionine or with the lipotropes, choline, methionine, and folate resulted in a cumulative probability of death with hepatocarcinoma and a final incidence of hepatocarcinoma similar to that of rats fed Diet 1. It is clear from comparison of this experiment with the one in which choline alone was supplemented that methionine exerted the major effect on hepatocarcinogenesis by DENA. This is consistent with the biochemical evidence of interactions between DENA and methionine. It is unlikely that the addition of folic acid was significant, since induction of folate deficiency in rats in the absence of antibiotic treatment or prevention of coprophagy did not occur or occurs to only a minor degree (14). Surprisingly, the addition of beef fat to Diet 1, rather than enhancing hepatocarcinogenesis by DENA, depressed it.

It can be concluded that deficiency of methionine rather than choline is responsible for the enhanced susceptibility of lipotrope-deficient rats to hepatocarcinogenesis and that the high-fat content of lipotrope-deficient diets does not contribute to the effect. The high level of cystine in Diet 2 compensates in part for the methionine deficiency and can be calculated to raise the effective methionine content to approximately 0.5%; the requirement is 0.67% (Table 2). Depression of hepatic S-adenosylmethionine is evidence that the rats are methionine deficient.8

Questions are raised by the appearance of sarcomas in animals fed the lipotrope- or amino acid-supplemented, high-fat diets but not in animals fed the control diet with added beef fat. In other experiments using DENA, we have found only 0 to 10% hemangioendothelial sarcomas, but this tumor characteristically appears in mice following treatment with DMNA or DENA (7, 29). The dietary effect may be the result of altered carcinogen metabolism in hepatic cells, or there may be a direct dietary influence on endothelial cells in the liver. In rats fed the severely deficient form of Diet 2, i.e., with no added choline, to induce fatty liver and cirrhosis, there was a marked increase in the turnover of hepatic endothelial cells which might enhance their susceptibility to carcinogens (20). However, one would have expected this to be reflected in tumorigenesis in rats fed Diet 2 rather than the supplemented diets. Studies are in progress using other carcinogens to determine whether this effect is specific for DENA.

REFERENCES


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