Folate Deficiency and Formiminoglutamic Acid Excretion during Chronic Diethylnitrosamine Administration to Rats

Lionel A. Poirier and V. Michael Whitehead

Institut du Cancer de Montréal, Hôpital Notre-Dame et Département de Biochimie, Université de Montréal (L. A. P.1. and Department of Haematology, and McGill University Medical Clinic, Montreal General Hospital (V. M. W.)). Montréal, Québec. Canada

SUMMARY

Elevated levels of the histidine catabolite formiminoglutamic acid were excreted into the urine of rats that were given both 0.01% diethylnitrosamine in their drinking water for 1 to 5 weeks and an injection of a loading dose of histidine. Similar histidine loading of control rats that received no carcinogen did not produce an elevation in urinary formiminoglutamic acid excretion. The elevation in urinary formiminoglutamic acid excretion caused by chronic diethylnitrosamine administration was prevented by high dietary levels of the methyl donors, methionine, betaine, and choline; high dietary levels of folate and vitamin B12, either alone or in combination, had no significant effect on the elevated formiminoglutamic acid excretion caused by diethylnitrosamine.

The elevated formiminoglutamic acid excretion caused by the administration of diethylnitrosamine for 3 weeks was associated with a decrease in the hepatic levels of the enzymes, formiminoglutamic acid transferase and urocanase, and with a decreased hepatic content of the higher conjugates of folate. Whereas dietary methionine administration completely prevented this decrease in the hepatic content of the higher conjugates of folate, dietary folate had no effect. Diets that contained elevated levels of methionine and choline also led to only a slight reversal of the decreased hepatic formiminoglutamic acid transferase activity caused by diethylnitrosamine. Thus the antagonistic effects on formiminoglutamic acid excretion by diethylnitrosamine and the methyl donors appeared to be mediated through the hepatic content of folate acid cofactors.

INTRODUCTION

Recent advances in chemical carcinogenesis have emphasized the importance of carbonium ions or electrophilic agents as the ultimate reactive forms for most, if not all, classes of chemical carcinogens (3, 6, 8, 10, 23, 38). However, the list of cellular nucleophiles implicated in the direct attack of such carbonium ions remains relatively limited, being restricted to certain constituents of the nucleic acids and proteins (3, 21, 23, 38) and to glycogen (11). Of the 4 essential dietary compounds involved in the transfer of 1-carbon units in vivo, 3 [methionine (19, 28, 32), folic acid (13), and vitamin B12 (16)] can behave as classical nucleophiles. Of these nucleophiles, only methionine reportedly is attacked in vivo by hepatocarcinogens (23, 24).

The limited quantities and the multiplicity of forms of both vitamin B12 and folic acid in the liver would make the direct demonstration of such an interaction in vivo quite difficult. Since each of the essential 1-carbon compounds or their derivatives alters the course of carcinogenesis (9, 18, 22, 24, 31, 33), the possible interrelationship between hepatocarcinogenesis and the metabolism of 1-carbon compounds is currently under investigation in these laboratories.

This paper is concerned with the effects of the chronic administration of diethylnitrosamine on the urinary excretion of the histidine catabolite FIGLU.3 Like other hepatocarcinogens, diethylnitrosamine appears to be metabolized to an electrophilic agent in the liver (8) and thus can be expected to attack nucleophilic sites in this organ. Studies show that urinary FIGLU excretion reflects a dietary or functional deficiency of the 3 essential 1-carbon nucleophiles, methionine, folic acid, and vitamin B12 (1, 2, 14, 20, 36, 37). A block in the catabolism of FIGLU is generally assumed to reflect low hepatic levels of H4-folate but may also reflect low hepatic levels of FIGLU transferase (1).

In both cases, the excess FIGLU is excreted into the urine. The tissue levels and activity of the folic acid cofactors appear to be regulated by nutritional status of the animal with respect to folic acid (1), vitamin B12 (2, 12, 37), choline (5), and methionine (2, 12, 25, 37). The hepatic levels of FIGLU transferase are controlled by dietary methionine (37), vitamin B12 (32, 37), and choline (29). Thus, elevated FIGLU excretion can act as a sensitive indicator of a metabolic abnormality of one or more of the essential 1-carbon compounds.

MATERIALS AND METHODS

Animals and Diets. Young, adult male Wistar rats (175 to 250 g) (Canadian Breeders, Inc., St. Constant, Quebec,

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2 Present address: National Cancer Institute, NIH, Bethesda, Md. 20014.
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2 The abbreviations used are: FIGLU, N-formimino-L-glutamic acid; H4-folate, tetrahydrofolic acid; FIGLU transferase, N-formimino-L-glutamate:tetrahydrofolic acid 5-formiminotransferase (EC 2.1.2.5).
Canada) were used in these experiments. Upon their arrival at this laboratory, the animals were housed (5/cage) in plastic, wire screen-topped cages containing soft wood shavings (Bran de Scie, Montreal, Quebec, Canada). Food (Micro-mix laboratory chow, Purina Ralston of Canada, Ltd., Woodstock, Ontario, Canada) and water were available ad libitum. After 1 week, groups of rats were given either 0.01% diethylnitrosamine (Eastman Organic, Rochester, New York, N. Y.), which was added to the drinking water for 1 to 5 weeks, or unsupplemented drinking water; the diethylnitrosamine was administered in aluminum-coated bottles. Where appropriate, DL-methionine and folate acid (Matheson, Coleman and Bell, East Rutherford, N. J.), choline chloride and vitamin B₁₂ (General Biochemicals, Inc., Chagrin Falls, Ohio), and betaine hydrochloride (Eastman Organic Chemicals, Rochester, N. Y.) were added to the Micro-mix diets of the diethylnitrosamine-treated and control groups, at levels of 15,000, 40, 10,000, 0.5, and 11,000 ppm, respectively.

Urine Collection and FIGLU Analyses. At 0, 1, 3, and 5 weeks, the rats from each group were weighed and given i.p. injections of L-histidine (Eastman Organic Chemicals) (225 μmoles/100 g body weight; 225 μmoles/ml 0.9% NaCl solution). The histidine injections were routinely performed at 3:00 p.m. The urine samples were collected for 18 hr, and the FIGLU excretion values were determined as previously described (35). After urine collection, the rats were returned to the dietary and carcinogenic regimen under investigation for subsequent histidine injections. The experiments were terminated at 5 weeks.

Folate Determinations. After 3 weeks of diethylnitrosamine, folate, and methionine administration, the rats were sacrificed by decapitation and exsanguinated. Their livers were quickly excised, weighed, and placed on ice. The livers were then homogenized (Polytron homogenizer; Kinematic, Lucerne, Switzerland) with 9 volumes of ice-cold 0.1 M phosphate buffer, pH 7.0, containing 8.24 mM ascorbic acid. The homogenates were then placed in a water bath for 5 min at 100° to precipitate the proteins; then the samples were cooled in ice and centrifuged at 2000 rpm for 15 min in an International Model PR-2 refrigerated centrifuge. The supernatants were collected and stored at 5°; their folate contents were determined (before and after conjugase treatment) with Lactobacillus casei, as previously described (39).

Enzyme Assays. The hepatic levels of 3 histidine-catabolic enzymes were determined in rats (9 to 12/group) that had received diethylnitrosamine, either alone or with 1.5% methionine or 1.0% choline for 3 weeks; these levels were also determined in control rats. After receiving diethylnitrosamine for 3 weeks, the rats were given injections of histidine, and their urine was collected for FIGLU analysis, as described above. At the end of the urine collection period (9:00 a.m.), the rats were sacrificed, their livers were excised and homogenized, and the homogenates were centrifuged at 100,000 X g for 1 hr, as previously described (29). The supernatants were stored at -20° for subsequent enzyme analysis. Histidase (L-histidine ammonia lyase, EC 4.3.1.3), urocanase, and FIGLU transferase all were assayed by previously described procedures on the Gilford Model 240 spectrophotometer (27, 34). The urocanic acid, FIGLU, and H₄-folate used in the assays were of the highest purity available and were purchased from Sigma Chemical Company, St. Louis, Mo.

RESULTS

Biological. The administration of 0.01% diethylnitrosamine in drinking water to rats for 1 to 5 weeks led to marked symptoms of toxicity. The diethylnitrosamine-treated rats did not gain a significant amount of weight during the 5-week experimental period, whereas the body weights of rats that received no diethylnitrosamine increased by 68 to 101 g. The high dietary levels of methionine, choline, folic acid, and vitamin B₁₂ appeared to have no significant effect on the body weights of the animals, regardless of the presence or absence of diethylnitrosamine in their drinking water. The liver weights of rats that received diethylnitrosamine for 3 weeks were significantly less than the liver weights of control rats (5.5 ± 0.2 versus 8.8 ± 0.3). The addition of methionine and choline to the diets of rats given diethylnitrosamine did not affect the diminished liver weights caused by the carcinogen.

Biochemical. As illustrated in Table 1, the administration of 0.01% diethylnitrosamine in drinking water to rats for 1 to 5 weeks resulted in a significant increase in urinary FIGLU excretion following a loading dose of histidine. At no time was there a significant change noted in the FIGLU level in the urine of control rats. The addition of 1.5% methionine to the diets of rats given diethylnitrosamine prevented completely the rise in FIGLU excretion (Tables 1 and 2). High dietary levels of folic acid and vitamin B₁₂ appeared to have no significant effect on the diethylnitrosamine-induced rise in FIGLU excretion (Table 1). A diet containing high levels of both folic acid and vitamin B₁₂ similarly exerted no effect on the elevated FIGLU excretion caused by diethylnitrosamine (Table 1). When a loading dose of histidine was injected into rats that received 1.1% betaine chloride in their diets (equimolar to 1.0% choline chloride), as well as diethylnitrosamine in their drinking water for 3 weeks, a normal urinary FIGLU level of 1.2 μmoles/100 g body weight was observed. In the same experiment, similarly treated control rats excreted 2.8 μmoles FIGLU/100 g body weight, while rats treated with diethylnitrosamine alone excreted 14.3 μmoles FIGLU/100 g body weight. Thus a 3rd physiological methyl donor greatly diminished the rise in FIGLU excretion observed during the administration of diethylnitrosamine.

Dietary supplementation with 1-carbon compounds appeared to produce other shifts in FIGLU excretion (Table 1). High dietary levels of methionine and choline led to slight but statistically significant decreases in FIGLU excretion by rats that received no diethylnitrosamine. When diethylnitrosamine was administered to rats that ingested high-methyl diets, urinary FIGLU levels returned to normal (Table 1). Similarly, rats given diets containing high levels of folate, but no carcinogen, excreted slightly less FIGLU than did the corresponding control rats. The physiological significance of these observations remains obscure. When rats were given both diethylnitrosamine and folate for 3 and 5 weeks, their urinary
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Table 1

**FIGLU excretion during chronic diethylnitrosamine administration to rats receiving methionine, choline, folate, and vitamin $B_{12}$**

Diethylnitrosamine was administered in the drinking water at a level of 0.01%; methionine, choline, folate, and vitamin $B_{12}$ were administered in the diet at levels of 15,000, 10,000, 40, and 0.5 ppm, respectively. All compounds were given for 1 to 5 weeks.

<table>
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<tr>
<th>Group</th>
<th>Weeks of feeding</th>
<th>Mean</th>
<th>Lower</th>
<th>Upper</th>
<th>95% confidence limits</th>
<th>Mean</th>
<th>Lower</th>
<th>Upper</th>
<th>95% confidence limits</th>
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<tr>
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<td>2.3</td>
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<td>0.8</td>
<td>1.9</td>
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<td>5</td>
<td>1.8</td>
<td>1.1</td>
<td>2.9</td>
<td>13.8</td>
<td>7.8</td>
<td>27.6</td>
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<td>3.2</td>
<td>8.9</td>
<td>17.0</td>
<td>6.0</td>
<td>46.3</td>
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<td>0.2</td>
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<td>32.7</td>
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<td>44.3</td>
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<tr>
<td></td>
<td>5</td>
<td>1.6</td>
<td>1.3</td>
<td>2.4</td>
<td>33.0</td>
<td>27.7</td>
<td>39.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ FIGLU excretion values have a positive asymmetrical distribution, which causes the skewness seen in the 95% confidence limits.

Table 2

**Urinary FIGLU and hepatic histidase, urocanase, and FIGLU transferase in rats given 0.01% diethylnitrosamine in the drinking water and dietary methionine and choline for 3 weeks**

After 3 weeks on each of the dietary regimens listed, the rats were given injections of 225 µmoles histidine per 100 g body weight, and their urine was collected for 18 hr. The FIGLU and enzyme assays were performed as described in "Materials and Methods." Each value represents the combined results of 3 experiments with 9 to 12 rats in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>95% confidence limits</th>
<th>Hepatic enzyme level (µmoles/hr/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.E.</td>
<td>Histidase</td>
</tr>
<tr>
<td>Control</td>
<td>2.0 ± 1.5</td>
<td>29.7 ± 1.5</td>
</tr>
<tr>
<td>Diethylnitrosamine</td>
<td>11.4 ± 8.8</td>
<td>27.0 ± 1.6</td>
</tr>
<tr>
<td>Diethylnitrosamine + 1.5%</td>
<td>1.8 ± 15.5</td>
<td>33.5 ± 1.5</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>3.5 ± 2.7</td>
<td>30.8 ± 1.6</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.E.
diets of pair-fed rats for 3 weeks, liver folate was determined as described in "Materials and Methods."

Following the administration of 0.01% diethylnitrosamine in the drinking water and of 40 ppm folate and 15,000 ppm methionine in the diets of pair-fed rats for 3 weeks, liver folate was determined as described in "Materials and Methods."

Table 3
The effects of dietary folate and methionine on the hepatic folate levels of pair-fed normal and diethylnitrosamine-treated rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver wt (g)</th>
<th>Hepatic folate (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without conjugase</td>
<td>With conjugase</td>
</tr>
<tr>
<td>Control</td>
<td>7.5 ± 0.7a</td>
<td>9.2 ± 0.7a</td>
</tr>
<tr>
<td>+ diethylnitrosamine</td>
<td>4.9 ± 0.3</td>
<td>8.2 ± 1.1</td>
</tr>
<tr>
<td>Folic acid</td>
<td>6.2 ± 0.1</td>
<td>10.9 ± 1.0</td>
</tr>
<tr>
<td>+ diethylnitrosamine</td>
<td>5.2 ± 0.2</td>
<td>7.7 ± 0.7</td>
</tr>
<tr>
<td>Methionine</td>
<td>6.5 ± 0.5</td>
<td>9.1 ± 0.6</td>
</tr>
<tr>
<td>+ diethylnitrosamine</td>
<td>5.4 ± 0.5</td>
<td>4.8 ± 0.1</td>
</tr>
</tbody>
</table>

a Mean ± S.E. of 5 to 6 rats.

significant (Table 2). The administration of high dietary levels of methionine and choline to rats given diethylnitrosamine for 3 weeks appeared to prevent increased FIGLU excretion and to reverse in part the decreased hepatic enzyme levels observed in such animals (Tables 1 and 2). High dietary levels of the 2 methyl donors, methionine and choline, appeared to give partial protection against the decrease in hepatic FIGLU transferase noted with the diethylnitrosamine-treated rats (p < 0.04 for diethylnitrosamine + methionine versus diethylnitrosamine; p < 0.05 for diethylnitrosamine + choline versus diethylnitrosamine). The administration of choline to the diethylnitrosamine-treated rats led to hepatic levels of urocanase that were significantly above the corresponding enzyme value in rats that had received diethylnitrosamine alone (p < 0.03). The feeding of methionine to diethylnitrosamine-treated rats appeared to give some protection against the decreased liver urocanase levels produced by diethylnitrosamine alone, but this difference was not statistically significant. In no case did the dietary administration of high levels of methyl donors completely reverse the altered enzyme levels produced by diethylnitrosamine.

The effects of 3 weeks of administration of diethylnitrosamine on the hepatic folate content of rats are described in Table 3. In control rats, diethylnitrosamine administration appeared to have no significant effect on the hepatic folate levels measured prior to conjugase treatment. However, the levels of the polyglutamate derivatives of folic acid, which are released by conjugase treatment, were 37% lower in the livers of the carcinogen-treated rats than they were in the livers of the control rats. The decrease in the hepatic polyglutamate levels of the diethylnitrosamine-treated rats was not affected by high dietary levels of folate but could be prevented by high levels of methionine (Table 3).

DISCUSSION

These experiments demonstrated that chronic diethylnitrosamine administration to rats produced a folate deficiency that could be prevented by the simultaneous administration of physiological methyl donors. Hepatic folate distribution and urinary FIGLU excretion are reported to be under the metabolic control of methyl donors (2, 12, 15, 25, 37). The shifts in urinary FIGLU excretion produced by diethylnitrosamine and methyl donor administration appeared to reflect an altered folate metabolism rather than a changed hepatic content of the histidine-catabolizing enzymes. Unless there was a marked difference in the activities of the histidine-catabolizing enzymes in vivo and in vitro, the hepatic levels of all of the enzymes studied appeared to be high enough to metabolize completely the injected histidine, even during diethylnitrosamine administration. Furthermore, the block in FIGLU catabolism noted during chronic diethylnitrosamine administration was associated with an altered content and distribution of the hepatic folate cofactors. The methionine-reversible decrease in the polyglutamate derivatives of folic acid noted in the livers of the diethylnitrosamine-treated rats also supports this proposal. Such a postulate appears reasonable in the light of findings that methyl donors influence the metabolism of vitamin $B_12$ and folic acid (2, 12, 15, 25, 37) and that vitamin $B_12$ deficiency leads to a secondary deficiency of folic acid (2, 37).

Previous workers have shown that a decreased liver content of both folic acid and vitamin $B_12$ occurs during chronic hepatocarcinogen administration (4, 7). The use of $L$. casei in the present experiments for measurement of folic acid did not permit a determination of the $H_4$-folate cofactors. However, previous studies indicated that methionine increases the availability of the $H_4$-folate cofactors in rat liver (25).

Our findings are in general agreement with previous observations that methyl donors often act as antagonists of the activity of such hepatocarcinogens as the aminoazo dyes (18, 22), ethionine (10), and aflatoxin (24). The antagonism between hepatocarcinogens and methyl donors is especially interesting in view of an apparent role of methionine in cellular differentiation (26). The decreased control by methyl donors of histidine catabolism could result from a diminished hepatic content of the methyl derivatives essential to folate metabolism. Such would be the result of a direct electrophilic attack of the activated carcinogen on methionine, for example. However, such an attack has been observed in vivo only with the aromatic amines and aminoazo dyes (22, 23). The nitrosamines have not yet been shown to attack methionine, although in vivo protein binding by these agents has been demonstrated (21). Carcinogen administration could also indirectly influence the hepatic content or metabolism of methyl compounds. Thus, Reid et al. (30) have reported an increased labeling of the methionine-methyl groups obtained from the livers of rats that were treated with $N,N$-dimethyl-4-aminooazobenzene and given injections of histidine-2,14C; also, the methylation of rRNA in the livers of rats treated with diethylnitrosamine was decreased, compared with the methylation of such RNA in normal liver (17). In addition, a greatly increased synthesis of the polyamines (40) or of phospholipids in the livers of rats treated with hepatocarcinogens could also be expected to produce a stress on the labile methyl pool.
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