Liver and Forestomach Tumors and Other Forestomach Lesions in Rats Treated With Morpholine and Sodium Nitrite, With and Without Sodium Ascorbate

Sidney S. Mirvish, Shahrokh Salmasi, Samuel M. Cohen, Kashinath Patil, and Ezzat Mahboubi

ABSTRACT—Administration to rats of ascorbate with morpholine and nitrite was previously shown to inhibit the liver tumor production and to enhance the induction of forestomach tumors, as compared to treatment with morpholine and nitrite. In a repetition of this experiment, 10 g morpholine/kg in the diet and 2 g sodium nitrite/liter in the drinking water were administered for life to male MRC-Wistar rats without (group 1) or with (group 2) 22.7 g sodium ascorbate/kg in the diet. Group 3 was untreated. Group 2 showed a lower liver tumor incidence with a longer latency than group 1, indicating a 78% inhibition by ascorbate of in vivo N-nitrosomorpholine (NMOR) formation. The incidence of forestomach papillomas was 3% in group 1, 38% in group 2, and 8% in group 3. The difference between groups 1 and 2 was not significant due to the shorter life-span of group 1. Group 1 and especially group 2 had more forestomach hyperplasia and hyperkeratosis than group 3.

Ascorbate might have enhanced induction of these lesions because of an action synergistic with that of NMOR. However, it is most likely that the lowered NMOR dose and concomitantly increased survival produced by the ascorbate were solely responsible for the increased incidence of forestomach papillomas and other lesions in group 2.

Materials and Methods

As before (4), morpholine and NaNO₂ (both American Chemical Society grade) were obtained from Fisher Scientific Co., Pittsburgh, Pa., and L-ascorbic acid was obtained from Sigma Chemical Co., St. Louis, Mo. The powdered and pelleted diet was Wayne Lab-Blox (Allied Mills Inc., Chicago, Ill.). Groups of outbred male MRC-Wistar rats from The Eppley Institute breeding colony were kept 4 cages and treated 5 days/week for life from 8 weeks of age. The treated rats received 10 g morpholine with or without 20.4 g ascorbic acid neutralized with NaOH (22.7 g sodium ascorbate)/kg powdered diet. Pelleted diet and tap water were administered to the controls for life and to the other groups on weekends and before the treatment started. Stock solutions in distilled water were prepared at 20 times the concentration in the food or drinking water, adjusted to pH 5 with HCl or NaOH (except for the nitrite), and stored at 6°C for not more than 4 weeks. The ascorbate stock solution was prepared with 408 g ascorbic acid/liter, corresponding after neutralization to 454 g sodium ascorbate/liter. Every 2 or 3 days, the NaNO₂ stock solution was diluted with distilled water and given ad libitum as drinking water, and

Abbreviations used: H & E = hematoxylin and eosin; NMOR = N-nitrosomorpholine.

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3 Animals were maintained under the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" by the Institute of Laboratory Animal Resources, National Research Council.
4 The Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 42d and Dewey Ave., Omaha, Nebr. 68105.
5 Eppley Institute and Pathology Department, University of Nebraska Medical Center.
6 Community Health Department, University of Nebraska Medical Center.
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b) distilled water, morpholine, or morpholine and ascorbate stock solutions were mixed by hand (with the use of gloves) with powdered diet to give a concentration of 10% water and given ad libitum to the rats.

Nitrite is stable under the conditions used (5). Morpholine was not determined in the diet because it is a stable amine that was unlikely to decompose. Ascorbate was determined in the diet by the 2,6-dichloroindophenol method (6). The recovery of sodium ascorbate (23 g/kg) added to the diets (some of which also contained 10 g morpholine/kg) was 78±6% (five analyses). Ascorbate recovery dropped a further 18±9% (five analyses) and 14±6% (five analyses) when these diets were stored for 2 days at room temperature or for 2 weeks at 6°C, respectively. The presence of morpholine did not affect these losses. In treated groups, the consumption per cage of water and (less accurately) food was measured for selected cages over 24-hour periods every week for the first 4 weeks and every 2 months for the next year. Rats were maintained until they were moribund or died. At autopsy, organs were fixed in 10% buffered Formalin, which was also injected into the stomach. Sections of the principal organs and of apparent tumors were prepared, stained with H & E, and examined.

If the base of the proliferating lesion in the forestomach was wider than its length, the term “hyperplasia” was applied. Hyperplasia and/or hyperkeratosis resulted in a thickening of the forestomach observed upon gross examination and appeared to be independent variables. Each of these lesions was graded as mild (grade 1), moderate (grade 2), or severe (grade 3). The forestomach stratum spinosum normally contained 3–5 cell layers (fig. 1). Thickening of this stratum to 10–15 (fig. 2), 15–25 (fig. 3), or more than 25 (fig. 4) cell layers was considered, respectively, mild, moderate, or severe hyperplasia. The normal stratum corneum of the forestomach mucosa was one-third to one-half as thick as the stratum spinosum (fig. 1). Hyperkeratosis was termed “mild,” “moderate,” or “severe” if thickening of the stratum corneum was, respectively, 2–5 (fig. 4), 5–10 (fig. 3), or more than 10 (fig. 5) times greater than normal. Lesions were termed “papillomas” only if they arose from the stratum spinosum with or without surface cornification and were attached to the gastric wall by a stalk.

Statistical analysis of the results was performed by the life-table method reviewed by Peto (7), based on the number of tumors observed in rats dying in successive 5-week intervals, relative to the number of rats alive (at risk) at the beginning of each time interval. Hyperplasia and hyperkeratosis were evaluated as follows: For each 5-week period, the total change was equated to the sum of the grades of the hyperplasia or hyperkeratosis of the rats dying in that interval. For each of these time intervals, the total change in the 2 groups under comparison was distributed to the 2 groups proportional to the animals at risk at the beginning of treatment. For each group, the sum of the change distributed in this way was then compared for significance with the observed change by the use of the life-table method (7). Results were considered significant when P was <0.05.

## RESULTS AND DISCUSSION

The food and water consumptions were, respectively, 52±10 g and 44±10 ml/rat/day in group 1 treated with morpholine plus nitrite and 52±16 g and 39±5 ml/rat/day in group 2 treated with morpholine plus nitrite plus ascorbate (mean ± SD for 7–10 measurements); they were not measured in control group 3. Ideally, control group 3 should have been treated with a diet containing 10% water and with distilled water as drinking water, as were groups 1 and 2. This omission is unlikely to have affected the results, because the moistened diet in groups 1 and 2 was stored for less than 3 days, too short for fungal or bacterial toxins to accumulate. The survival data are shown in table 1, and the principal results are given in table 2.

### Liver Tumors

Ninety-five percent of the rats in group 1 and 41% of those in group 2 developed liver tumors, most of which were hepatocellular carcinomas, often with lung metasizes. The controls (group 3) showed no liver tumors. Statistical analysis, which took account of the different survival periods, showed that liver tumor incidence was significantly higher than the control value in both groups 1 and 2 (P<0.01) and was significantly higher in group 1 than it was in group 2 (P<0.05).

In group 2, the liver tumor incidence was reduced by a factor of 0.43, and the tumor latency was increased by a factor of 1.96, relative to the position in group 1. Since treatments were given for life, the increased latency implied a corresponding increase in the NMOR dose produced in vivo. If liver tumor induction was proportional to NMOR dose, then this result indicates that in vivo NMOR production was inhibited 100(1 – 0.43/1.96) = 78% by addition of ascorbate to the diet. This compares with an estimated 56% inhibition in the previous study (4). The greater effectiveness of ascorbate in the present test was probably due to the higher ascorbate-to-nitrite ratio, resulting from the lower dose of nitrite used here.

The 95% liver tumor incidence in group 1 given morpholine and 0.2% NaNO₂ in the drinking water was substantially higher than the 65% incidence of these tumors observed previously (4), when morpholine and 0.3% NaNO₂ were administered. This finding suggested that the lower dose of endogenously produced NMOR was more efficient.

### Table 1.—Survival of experimental groups

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment</th>
<th>No. of rats surviving at wk:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>Morpholine + NaNO₂</td>
<td>39</td>
</tr>
<tr>
<td>2</td>
<td>Morpholine + NaNO₂ + ascorbate</td>
<td>37</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>39</td>
</tr>
</tbody>
</table>
Effects in the Esophagus

NMOR has induced a few tumors in the rat esophagus (8). However, examination of sections from the esophagi of 10 rats from each group revealed no abnormalities.

Forestomach Tumors

Only 1 squamous cell carcinoma was observed (in group 2), unlike the position in the previous test (4), in which an 18% incidence of these tumors was induced. The incidence of squamous cell papillomas (fig. 6) was 3% in group 1, 38% in group 2, and 8% in group 3. Statistical analysis showed no significant differences among the 3 groups (P > 0.05), though P for the comparison between groups 2 and 3 was 0.07. Hence we did not prove that the endogenously formed NMOR induced these tumors, although this was likely in group 2. The explanation for the lack of a significant difference between groups 1 and 2 lies in the fact that group 1 showed a mean survival of only 55 weeks, whereas the rats with papillomas in group 2 had a mean survival of 94 weeks. Hence the low tumor incidence in group 1 could be explained solely by their short life-span. It is likely that endogenously formed NMOR was solely responsible for the papillomas and that the ascorbate did not contribute directly to their development.

NMOR has not been reported to induce forestomach tumors in rats (4, 9), but the reported tests did not include dose-response studies, and we would expect forestomach tumors only when the dose was low enough for the liver tumor latency to be increased to about 2 years. Also, NMOR may have induced forestomach tumors because it was produced under the acidic conditions of the glandular stomach and diffused back into the forestomach. This system might induce more forestomach tumors than if NMOR were administered in food or drinking water.

In our previous test (4) the group given morpholine plus nitrite plus ascorbate showed a 54% incidence of forestomach tumors, and the group given morpholine plus nitrite did not show any forestomach tumors. In the report of that test (4), we did not analyze the results statistically. When we analyzed these results by the methods used in the current test, forestomach tumor incidence in the group given morpholine plus nitrite plus ascorbate was significantly (P < 0.001) greater than that in the untreated group but not greater than that in the group given morpholine plus nitrite. As in the present test, the latter finding is attributed to the early death due to liver tumors of the group given morpholine plus nitrite, since this situation did not allow sufficient time for forestomach tumors to develop.

We previously demonstrated that NMOR, administered in drinking water, induced liver tumors in MRC-Wistar rats, but their lifetime of 28 weeks was too short to expect forestomach tumors; in fact, the incidence of forestomach tumors was only 2% (4). We also observed an 18% incidence

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**Table 2.—Summary of tumor incidences**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1, morpholine + nitrite</th>
<th>Group 2, morpholine + nitrite + ascorbate</th>
<th>Group 3, untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective No.</td>
<td>39</td>
<td>37</td>
<td>39</td>
</tr>
<tr>
<td>Tumor-bearing animals</td>
<td>38</td>
<td>27</td>
<td>16</td>
</tr>
<tr>
<td>Rats with each type of lesion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any type of liver tumor</td>
<td>37</td>
<td>35</td>
<td>39</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>36</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Other types of liver tumor</td>
<td>3</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>Foregut squamous cell papilloma</td>
<td>7</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>Other tumors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forestomach hyperplasia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>14</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Grade 2</td>
<td>1</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Grade 3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>26</td>
<td>3</td>
</tr>
<tr>
<td>Forestomach hyperkeratosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>13</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Grade 2</td>
<td>5</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Grade 3</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>30</td>
<td>4</td>
</tr>
</tbody>
</table>

*Values are means ± SD.

1 Number of rats alive when first tumor-bearing animal of that group died.

28 with lung metastases.

5 with lung metastases.

1 cholangioma, 1 bile duct adenoma, and 1 hemangiosarcoma.

1 hemangioendothelioma.

Also, 1 transitional cell papilloma of forestomach.

Including 3 kidney tumors (2 adenocarcinomas, 1 transitional cell carcinoma, 68 wk).

Including 4 pituitary adenomas (80 wk), 3 adrenal pheochromocytomas (93 wk), 3 testicular (Leydig’s cell) tumors (97 wk), 2 pancreatic tumors (1 acinar cell adenoma, 1 ductular adenoma, 129 wk), and 1 forestomach squamous cell carcinoma (116 wk).

Including 6 pancreatic tumors (4 acinar cell adenomas, 2 islet cell adenomas, 68 wk), 4 adrenal pheochromocytomas (97 wk), 3 testicular (Leydig’s cell) tumors (97 wk), 2 pancreatic tumors (81 wk), and 2 testicular (Leydig’s cell) tumors (89 wk).

In the previous test (4) the group given morpholine plus nitrite plus ascorbate showed a 54% incidence of forestomach tumors, and the group given morpholine plus nitrite did not show any forestomach tumors. The reported test (4) did not include dose-response studies, and we would expect forestomach tumors only when the dose was low enough for the liver tumor latency to be increased to about 2 years. Also, NMOR may have induced forestomach tumors because it was produced under the acidic conditions of the glandular stomach and diffused back into the forestomach. This system might induce more forestomach tumors than if NMOR were administered in food or drinking water.

In our previous test (4) the group given morpholine plus nitrite plus ascorbate showed a 54% incidence of forestomach tumors, and the group given morpholine plus nitrite did not show any forestomach tumors. In the report of that test (4), we did not analyze the results statistically. When we analyzed these results by the methods used in the current test, forestomach tumor incidence in the group given morpholine plus nitrite plus ascorbate was significantly (P < 0.001) greater than that in the untreated group but not greater than that in the group given morpholine plus nitrite. As in the present test, the latter finding is attributed to the early death due to liver tumors of the group given morpholine plus nitrite, since this situation did not allow sufficient time for forestomach tumors to develop.
of forestomach tumors in MRC-Wistar rats of both sexes treated with 3 g NaNO₂/liter drinking water (5), so that some forestomach lesions in groups 2 and 3 may have been caused by the nitrite. We have not administered morpholine alone, ascorbate alone, or ascorbate plus nitrite to rats, but in our earlier studies on lung adenoma induction in strain A mice by combinations of morpholine, nitrite, and ascorbate, groups treated with morpholine alone (6.33 g/kg diet) and sodium ascorbate alone (23 g/kg diet) showed negative results (10).

The 8% incidence of forestomach papillomas in group 3 was similar to that found previously in our untreated rats; e.g., the incidence of these tumors was 4% in the untreated male rats in (4).

Hyperplasia and Hyperkeratosis of the Forestomach

Group 2 showed an 84% greater incidence of hyperplasia and a 65% greater incidence of hyperkeratosis than group 1. Group 2 showed more lesions graded 2 or 3 than did group 1, and the statistical evaluation took these gradings into account. The incidence and severity of the two types of lesions were similar, and the evaluation was applied equally to both lesion types. Both groups 1 and 2 had higher incidences of hyperplasia and hyperkeratosis than did control group 3, with \( P<0.01 \) for group 1 and \( P<0.05 \) for group 2. The incidence of these lesions in group 2 was greater than that in group 1, with \( P=0.06 \). We concluded that the treatment with morpholine plus nitrite induced hyperplasia and hyperkeratosis of the forestomach both in the presence and absence of ascorbate and that the increased incidence and severity of these lesions in the presence of ascorbate were mostly due to the longer survival of the group 2 rats, but they may also have been due to a specific effect of the ascorbate. If there was such a specific effect, this was probably synergistic with the endogenously produced NMOR or possibly with the administered morpholine and/or nitrite.

An alternative explanation is that the lower dose of NMOR in group 2 was more effective in inducing hyperplasia and hyperkeratosis than the higher dose of NMOR in group 1, i.e., that the ascorbate had no direct effect. A test of ascorbate administered in the diet together with various levels of preformed NMOR in drinking water might settle this question, as well as the question of whether ascorbate contributes to the development of forestomach papillomas.

REFERENCES


Figures 1-6.—Forestromachs of rats from groups 1-3. Bars=200 μm. All sections were stained with H & E.

Figure 1.—Normal forestomach.
Figure 2.—Mild hyperplasia with mild hyperkeratosis. Prominent parakeratosis is also present.
Figure 3.—Moderate hyperplasia with moderate hyperkeratosis.
Figure 4.—Severe hyperplasia with mild hyperkeratosis.
Figure 5.—Moderate hyperplasia with severe hyperkeratosis.
Figure 6.—Squamous cell papilloma.