Effects in Rats of Sodium Chloride on Experimental Gastric Cancers Induced by N-Methyl-N' nitro-N-nitrosoguanidine or 4-Nitroquinoline-1-oxide

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SUMMARY—The effects were studied of NaCl on the production of gastric carcinomas by N-methyl-N' nitro-N-nitrosoguanidine (MNNG) and by 4-nitroquinoline-1-oxide (NQO) in male Wistar rats. Nine groups of rats were treated as follows: Group 1 was given 50 mg MNNG/liter and 6 g NaCl solution/liter to drink and was fed a stock diet supplemented with 10% NaCl. Group 2 received 1 ml saturated NaCl once a week and 50 mg MNNG/liter to drink. Group 3 was treated with MNNG alone. Group 4 was given a solution of 1 mg NQO once a week and fed a stock diet supplemented with 10% NaCl. Group 5 received a solution of 1 mg NQO saturated with NaCl. Group 6 was given NQO alone. Groups 7 and 8 were given NaCl alone. Group 9 was untreated. Adeno­carcinomas developed in the glandular stomach in group 2 at a significantly higher incidence than in group 3. Poorly differ­entiated adenocarcinomas of the glandular stomach were de­tected in only groups 1 and 2. One poorly differentiated adenocarcinoma metastasized to the lymph nodes. A high incidence of squamous cell carcinomas of the forestomach was found in groups 4 and 5. No malignant tumors were seen in groups 6–9. NaCl given alone had no apparent carcinogenic­ity in rats but, when administered with MNNG or NQO, it en­hanced the carcinogenic effects of MNNG and NQO in the stomach.—J Natl Cancer Inst 55: 101–106, 1975.

STOMACH CANCER occurs at the highest incidence in the world in Japan (1). From necropsy studies, Stei­ner (2) was impressed by the higher frequency of stomach cancer in Japanese than in other ethnic groups in the United States. Haenszel et al. (3) found that first­generation immigrants from prefectures in Japan with a population having a high risk of developing stomach cancer still had a high risk in Hawaii, whereas their sec­ond­generation offspring did not. Therefore, the mortal­ity rate from gastric cancer is not closely linked with ethnic groups. The geographic comparison of Sato et al. (4) indicated that the incidence of gastric cancer paral­leled the amount of salted foods consumed. Hirayama (3) also suggested a close association between stomach cancer and an excess intake of highly salted pickles.

Adenocarcinomas in the glandular stomach of rodents were recently induced by various kinds of carcinogens (6–14). Attempts to induce gastric cancer in animals by salted foods were unsuccessful (15). However, NaCl treatment greatly increased the induction of squamous cell carcinomas by 4-nitroquinoline-1-oxide (NQO) in the mouse forestomach (16), and hypertonic saline enhanced the uptake of 7,12-dimethylbenz[a]anthracene by the gastric wall (17). We, therefore, studied the effects of NaCl on the induction of carcinomas by N-methyl-N' nitro-N-nitrosoguanidine (MNNG) and NQO in the rat stomach.

MATERIALS AND METHODS

The gastric carcinogens were MNNG and NQO. MNNG was obtained from the Aldrich Chemical Company, Inc. (Milwaukee, Wis.), and NQO as well as NaCl were commercial products from Nakarai Chemical, Ltd. (Kyoto, Japan).

A total of 160 male Wistar rats (initially weighing 200 g) were treated as follows:

Group 1.—Twenty rats were given ad libitum 50 mg MNNG/liter and 6 g NaCl solution/liter to drink and were fed the stock diet (Oriental MF, Oriental Yeast Co., Ltd., Tokyo, Japan) supplemented with 10% NaCl.

Group 2.—Twenty rats were given every day to drink 50 mg MNNG/liter and once a week 1 ml saturated NaCl solution (29.0%) by intragastric tube and were fed the stock diet.

Group 3.—Thirty rats were given 50 mg MNNG solution/liter to drink and were fed the stock diet.

Group 4.—Twenty rats were given once a week a solution of 1 mg NQO in 1 ml of 20% ethanol by intragastric tube and were fed the stock diet supplemented with 10% NaCl.

Group 5.—Twenty rats were given once a week a solution of 1 mg NQO in 1 ml of 20% ethanol saturated with NaCl (18.0%) by intragastric tube and were fed the stock diet.

Group 6.—Twenty rats were given once a week a solution of 1 mg NQO in 1 ml of 20% ethanol by intragastric tube and were fed the stock diet.

Group 7.—Ten rats were given to drink a solution of 6 g NaCl/liter and were fed the stock diet supplemented with 10% NaCl.

Group 8.—Ten rats were given 1 ml saturated NaCl solution as in group 2 and were fed the stock diet.

Group 9.—Ten rats were given the stock diet and tap water.

Rats were housed 5 to a screen­bottomed cage (10.5 X 15.0 X 7.0 inches) under natural light in an air­conditioned room at 24° C; they were weighed weekly. The MNNG solution was kept in light­proof bottles and renewed every day from a stock solution (1 g/liter) prepared weekly. The rats were treated with MNNG or NQO for 20 weeks; then all were maintained on the stock diet and tap water. Only animals surviving for over 40 weeks were included in the results. All rats that died or that were killed when moribund were autopsied. The stomach and other organs were examined carefully and fixed in a 10% formaldehyde solution for histologic examination. Tissues were embedded in paraffin and stained with hematoxylin and eosin. Selected tissues were stained with alcian blue, periodic acid­Schiff, Mayer's mucicarmine, or van Gieson's agent.

The results were analyzed by the $\chi^2$ test.

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## RESULTS

The average body weights of rats in all groups gradually increased during treatment (Table 1). Groups 1 and 4 had the least increase in weight, but 10 weeks after the treatment their average body weights increased to as much as those of the other groups. The average survival times (Table 1) of the groups differed slightly. Almost all rats in groups 7–9 were still alive after 85 weeks, at which time they were killed.

The intake of drinking water by all rats fed the stock diet supplemented with 10% NaCl was 1.2–1.5 times that of rats on the stock diet. Polyuria and polydipsia developed but disappeared after the treatment period. Rats given 1 ml of the saturated NaCl solution once a week had no polyuria or polydipsia. Hypernatremia did not induce significant histopathologic changes in any organs.

The total intakes of MNNG by animals in groups 2 and 3 were the same, whereas the total intake of MNNG in group 1 was 1.2–1.5 times more. The exact intakes of MNNG are unknown, because MNNG was given in the drinking water ad libitum. The total NQO intake per rat in groups 4–6 was 20 mg.

### Stomach Tumors

The incidence of gastrointestinal tumors in each group is summarized in Table 2. In groups 1–3 treated with MNNG, tumors developed mainly in the glandular stomach; however, in groups 4–6 given NQO, almost all tumors developed in the forestomach. No tumors were found in groups 7–9, which did not receive the carcinogens.

#### Forestomach

Tumors of the forestomach usually were large papillary and hyperkeratotic masses occupying the lumen, with nodules extending into the serosa. Multiple, small papillomas of the forestomach were often observed.

All tumors of the forestomach were of squamous cell origin (fig. 5). The incidence of squamous cell carcinoma of the forestomach in groups 4 and 5 differed significantly from that in group 6: 38.9% in group 4 ($P<0.01$) and 52.9% in group 5 ($P<0.01$), with rats in group 6 showing only small squamous cell papillomas of the forestomach. A squamous cell carcinoma metastasized to the intestinal lymph nodes (fig. 7) and liver in one rat in group 5.

#### Glandular stomach

Most tumors of the glandular stomach were in the lesser curvature of the pyloric region. They were polypoid nodules or ulcerative umbilicated nodules with elevated borders and appeared as smooth-surfaced globules extending into the serosa. Some adhered to the omentum and the liver.

The adenocarcinomas of the glandular stomach were of two histologic types: well differentiated and poorly differentiated. The well-differentiated tumors were composed of tubular, papillary, or cystic glandular structures (fig. 5). The poorly differentiated tumors were characterized by loss of the glandular arrangement. The anaplastic tumor cells showed polymorphism, increased basophilia of the cytoplasm, and hyperchromatic nuclei; some contained intracellular mucin or formed irregular tubular structures (fig. 4).

### Table 1.—Changes in survival time and body weight of rats treated with MNNG or NQO and NaCl

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Number of rats</th>
<th>Average survival time (wk)</th>
<th>Body weight (g)</th>
<th>Initial</th>
<th>After 40 wk</th>
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<tbody>
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<td>71</td>
<td>185.6</td>
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<td>80</td>
<td>188.0</td>
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<td>10</td>
<td>85</td>
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<td>445.8</td>
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<tr>
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<td>10</td>
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<td>187.1</td>
<td>453.0</td>
<td></td>
</tr>
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<td>10</td>
<td>85</td>
<td>198.0</td>
<td>460.4</td>
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</table>

### Table 2.—Incidence of tumors in rats treated with MNNG or NQO and NaCl

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Number of effective rats</th>
<th>Fore stomach</th>
<th>Glandular stomach</th>
<th>Small intestine</th>
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</thead>
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<tr>
<td></td>
<td>Papilloma</td>
<td>Squamous cell carcinoma</td>
<td>Well-differentiated adenocarcinoma</td>
<td>Poorly differentiated adenocarcinoma</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>11</td>
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<td>0</td>
<td>0</td>
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<tr>
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<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

* Numbers in brackets indicate number with metastatic changes.
* Sarcomas in the glandular stomach plus adenocarcinomas or squamous cell carcinomas.
* One was a sarcoma of the colon.
reaction (fig. 2). The metastatic lesions were muconodu­lar adenocarcinomas with extracellular mucin, and the cells containing mucin in the mucocellular adenocarcino­

noma-induced by NQO with and without NaCl (groups 4 and 5 vs. group 6). The present results confirm that NaCl alone is not carcinogenic; however, NaCl enhanced the carcinogenic effects of MNNG and NQO in the stomach of rats.

All rats with a sarcoma of the glandular stomach also had an adenocarcinoma of the glandular stomach, ex­cept for 1 rat in group 4 that had a squamous cell carcino­noma of the forestomach. Two rats in group 2 had me­tastases to the spleen, and 1 rat in group 4 had metastases to the pancreas.

Nontumor Areas of Stomach

The histopathologic changes in nontumor areas of the stomach are shown in table 3. Pyloric gland metaplasia in the fundic mucosa and hyperplastic and atrophic le­sions of the pyloric mucosa were detected mainly in the groups given MNNG. No intestinal metaplasia of the glandular stomach was seen in any group. Multiple focal hyperplastic changes of the squamous epithelium of the forestomach associated with hyperkeratosis were observed in the groups given NQO.

Other Organs

Some tumors were found in the small and large intestines. Four adenocarcinomas and 5 sarcomas of the small intestine were found in group 1. Three of the adenocarcinomas and 2 of the sarcomas metastasized directly to the liver, pancreas, and omentum. One adenocarcinoma and 5 sarcomas of the small intestine were found in group 2; but they did not metastasize. One adenocarcino­noma and 5 sarcomas of the small intestine with no me­tastases were observed in group 3. One sarcoma of the large intestine also was seen in group 3. No intestinal tumors were found in groups 4–9. Moreover, no malignant tumors were detected outside the gastrointestinal tract in any group.

DISCUSSION

The difference in the incidence of gastric cancer induced by MNNG with and without saturated NaCl so­lution (group 2 vs. group 3) was statistically significant (P<0.05). Results (P<0.01) were similar with regard to the difference in the incidence of squamous cell carci­nomas induced by NQO with and without NaCl (groups 4 and 5 vs. group 6). The present results confirm that NaCl alone is not carcinogenic; however, NaCl enhanced the carcinogenic effects of MNNG and NQO in the stomach of rats.

The mechanism of the effect of NaCl is still un­known. From histologic studies on gastric cancer, Mu­rekami (18) suggested that repeated injuries of the gas­tric mucosa may be one factor causing cancer. However, although erosion and ulceration of the glandular stom­ach and forestomach were seen after administration by intragastric tube of 1 ml of a saturated solution of NaCl to rats, these foci rapidly healed and did not induce gas­tric cancer (Tatematsu M: Unpublished data). Sato et al. (15) also observed that injury of the gastric mucosa of mice by highly salted foods did not induce gastric cancer. Conversely, little erosion and no ulceration of the stomach were found in rats given 50 mg MNNG solution/liter, which induced a high incidence of stom­ach carcinomas (19). Therefore, erosion or ulceration per se in the stomach may not evolve into gastric carci­noma. However, such lesions may become cancerous in the presence of a carcinogen, even at a low concentra­tion.

The effects of NaCl in enhancing the carcinogenic activities of NQO and MNNG bear a curious resemblance to the effects of surface-active agents. Previous papers (13, 20) from this laboratory reported that administration by intragastric tube of NQO in dilute ethanol, combined with the strong surfactant alkylbenzenesulfonate to rats, induced undifferentiated adenocarcino­mas of the glandular stomach with metastases. More­over, many well-differentiated and some undifferentiated adenocarcinomas developed in the glandular stomach with metastases when MNNG, combined with strong surfactants, was given orally to rats (21, 22).

It has been proposed that the mucosal barrier in the stomach is important in preventing the induction of stomach carcinomas in rats (23–25). However, the above results suggest that carcinogens administered in the presence of surfactants may be able to penetrate the protective mucosal barrier of the stomach mucosa.

The gastric mucosa contains several kinds of acid mu­copolysaccharides (26, 27), the viscosity of which decreases in the presence of NaCl (28, 29). Thus possibly NaCl decreases the viscosity of the gastric mucous and so reduces the protective mucous barrier. If so, use of NaCl as a vehicle for MNNG would reduce the protective mu­

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Forestomach</th>
<th>Fundic mucosa</th>
<th>Glandular stomach</th>
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<tbody>
<tr>
<td></td>
<td>Focal hyperplasia</td>
<td>Erosion or ulcer</td>
<td>Pyloric gland metaplasia</td>
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<tr>
<td>1</td>
<td>±</td>
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<td>±</td>
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<td>9</td>
<td>±</td>
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</table>

* The changes were divided in 4 grades as follows: −, no change; ±, very slight change; +, slight change; ++, moderate change; and ++++, marked change.
uous barrier of the glandular stomach and allow direct contact of MNNG with the gastric mucosa. Capoferro and Torgersen (17) reported that 2 moles of hypertonic saline enhanced the uptake of 7,12-dimethylbenz[a]anthracene by both the forestomach and glandular stomach. They suggested that this might be due to removal of the mucous layer of the glandular stomach and loss of the superficial epithelial cells of the forestomach. The forestomach is not protected by mucins as the glandular stomach is, so that loss of superficial squamous cells after administration of hypertonic saline probably explains the high incidence of squamous cell carcinoma in groups 4 and 5.

The MNNG intake by rats in group 1 was more than that by rats in groups 2 and 3, but the incidence of adenocarcinoma of the glandular stomach in group 1 was not significantly higher than that in group 3 (P<0.3). Two possible reasons exist for this lack of a significant difference: 1) MNNG may be degraded by NaCl because MNNG is more stable in distilled water than in tap water (30), and 2) the concentration of 10% NaCl in the stock diet was not high enough to reduce the mucous barrier. The only poorly differentiated adenocarcinoma with metastases was found in group 1, and the incidence of tumors of the small intestine was higher in group 1 than in group 3 (P<0.1). These results suggest that lack of a significant difference in the incidence of adenocarcinoma of the glandular stomach in groups 1 and 3 was not due to degradation of MNNG by NaCl but to insufficient reduction of the mucous barrier by NaCl.

NaCl might act as a "promotor" according to Beralbum's initiation-promotion concept (31). However, if NaCl is a promotor, then the incidence of gastric cancer should have been higher in group 1 than in group 2 and in group 4 than in group 5, because the intakes of NaCl in the food in groups 2 and 5 were larger than the amount of NaCl given weekly by intubation to groups 1 and 4. However, the opposite results were observed. Thus NaCl does not seem to be a promotor but does seem to enhance the penetration of the carcino gens.

Related to these results are the results found in a study in which some pickled vegetables eaten by Japan ese, e.g., white radish misozuke, were found to contain 30% NaCl, a salt concentration as high as that in the saturated NaCl solution (4). This concentration is sufficient to induce not only reduction of the mucous barrier but also erosion or ulceration of the stomach.

REFERENCES

(2) STEINER PE: Cancer: Race and Geography. Baltimore, Williams & Wilkins, 1954
(5) HIRAYAMA T: Epidemiology of stomach cancer. Gann Monogr 11:5-19, 1971
(9) MORI K, OHTA A: Carcinoma of the glandular stomach of mice by 4-hydroxyanilinoquinoline 1-oxide. Gann 58:551-554, 1967
(10) MORI K: Carcinoma of the glandular stomach of mice by instillation of 4-nitroquinoline 1-oxide. Gann 58:589-593, 1967
(20) TAKAHASHI M: Effect of alkylbenzenesulfonate as a vehicle on the uptake of tritiated 7,12-dimethylbenz[a]anthracene by the gastric mucosa. Scand J Gastroenterol 9:343-349, 1974
(22) TAKAHASHI M, FUKUSHIMA S, SATA H: Carcinogenic effect of N-methyl-N-nitro-N-nitrosoguanidine with various kinds of surfactants in the glandular stomach of rats. Gann 64:211-218, 1975
(28) LEY R: The mucin histochemistry of normal and neoplastic gastric mucosa. Lab Invest 14:2080-2100, 1965
FIGURE 1.—Section of tumor in glandular stomach (group 1). Hematoxylin and eosin (H & E). × 4

FIGURE 2.—Higher magnification of tumor in glandular stomach in figure 1. Poorly differentiated adenocarcinoma consists of mucin-secreting tumor cells. Alcian blue and periodic acid-Schiff. × 200

FIGURE 3.—Metastatic mucinodular adenocarcinoma producing extracellular mucin in lymph node (group 1). Alcian blue and periodic acid-Schiff. × 100
FIGURE 4.—Poorly differentiated adenocarcinoma of glandular stomach (group 2). H & E. × 200

FIGURE 5.—Well-differentiated adenocarcinoma of glandular stomach (group 3). H & E. × 100

FIGURE 6.—Invasive growth of squamous cell carcinoma of forestomach into muscle layer of glandular stomach (group 4). H & E. × 100

FIGURE 7.—Metastasis of squamous cell carcinoma of forestomach to intestinal lymph node (group 5). H & E. × 100