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Carcinogenicity of Methylurea or Morpholine in Combination with Sodium Nitrite in a Rat Multi-organ Carcinogenesis Bioassay

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For carcinogenic risk assessment of combinations of *N*-nitroso precursors in man, the effects of feeding methylurea (MU) or morpholine (Mor) plus sodium nitrite ($NaNO_2$) were investigated using a multi-organ carcinogenesis model. In experiment 1, to initiate multiple organs, groups of 10 or 20 male F344 rats were treated with 6 carcinogens targeting different organs. Starting a week after completion of this initiation phase, animals were given 0.1% MU or 0.5% Mor in their food and/or 0.15% $NaNO_2$ in their drinking water for 23 weeks. The induction of tumors and/or preneoplastic lesions in the forestomach and esophagus was significantly increased in the group receiving MU plus $NaNO_2$. The numbers and areas of liver glutathione *S*-transferase placental form (GST-P)-positive foci were significantly elevated with MU or Mor plus $NaNO_2$. Experiment 2 was conducted to assess formation of *N*-nitroso compounds in the stomach, and to detect DNA adduct generation in target organs by immunohistochemical staining. Groups of 5 or 14 animals were starved overnight, then given 0.4% MU or 2.0% Mor in the diet, or basal diet alone for 1 h. Then $NaNO_2$ or distilled water was given intragastrically. The mean gastric *N*-methyl-*N*-nitrosourea yield in the MU plus $NaNO_2$ group was 7700 μg at 2 h after combined administration. The mean *N*-nitrosomorpholine yield in the group given Mor plus $NaNO_2$ was 6720 μg. Immunohistochemically, N7-methyldeoxyguanosine-positive nuclei were evident in the forestomach epithelium at 8 h after the combination treatment with MU plus $NaNO_2$.

Key words: Methylurea — Morpholine — Sodium nitrite — Multi-organ carcinogenesis — *N*-Nitroso compound

The *N*-nitroso compounds are well known strong carcinogens, closely related to food and nutrition, which constitute one of the most important risk factors in man.[1)] Precursor nitrites and amines are contained in many common foods,[2)] and can react under the acidic conditions of the stomach to form nitrosamine or related carcinogens.[3–6)] Thus, Mirvish *et al.* reported that treatment of Swiss mice with MU or Mor (in the diet) and $NaNO_2$ (in the drinking water) resulted in an increased incidence of lung adenomas when compared to control mice.[7,8)] Shank and Newberne conducted similar experiments, in which rats and hamsters fed Mor plus $NaNO_2$ developed hepatocellular carcinomas and angiosarcomas in the liver.[9)] Koestner and Denlinger further demonstrated that the combination of MU and $NaNO_2$ induced neurogenic and lymphoid neoplasms in rats which were treated with these agents for 2 years.[10)] The compound formed from MU and $NaNO_2$ is MNU, which is known to act as a carcinogen in not only the nervous tissues, but also the digestive system, thymus, etc. Inherently, the target organs for amine plus nitrite are the same as for the nitroso compound. However, there has hitherto been no report about tumor induction involving the digestive system, thymus, etc. *in vivo*. Furthermore, the whole question of target organ specificity with this route of carcinogen exposure has received only limited attention. Therefore, the present study was performed to cast light on this question, as well as which combinations impose the greatest risk.[2,11,12)]

For the present study, MU or Mor and $NaNO_2$ were chosen as precursors considered to be high risk factors for human carcinogenesis.[2)] MU is formed from creatine-containing foods, such as smoked or dried bonito fish

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Abbreviations used: MU, methylurea; Mor, morpholine; $NaNO_2$, sodium nitrite; MNU, *N*-methyl-*N*-nitrosourea; NNM, *N*-nitrosomorpholine; DEN, diethylnitrosamine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DMH, 1,2-dimethylhydrazine; BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; DHPN, 2,2'-dihydroxy-di-*n*-propylnitrosamine; ENUR, ethylnitrosourethane; GST-P, glutathione *S*-transferase placental form; PAPG, pepsinogen 1 (Pg1) altered pyloric glands; PN, papillary or nodular; O^6-medG, O^6-methyldeoxyguanosine; N7-medG, N7-methyldeoxyguanosine.

products and fried bacon, by nitrosation-denitrosation,[2,13] so that a contribution to *in vivo* production of carcinogens and the etiology of human gastric cancer, particularly in Japan, is very probable.[3] NNM, which is yielded by the reaction of Mor with $NaNO_2$, is a potent carcinogen which induces tumors of the liver in mice and rats.[14] Mor has gained widespread acceptance as a cheap solvent for resins, waxes, casein and dyes.[15] Morpholine fatty acid salts are also used as surface-active agents and emulsifiers, often being used for coating fresh fruits to protect them from deterioration.[16] Related compounds are used as corrosion inhibitors, antioxidants, plasticizers, viscosity improvers, insecticides, fungicides, herbicides, local anesthetics and antiseptics. $NaNO_2$, the best-known dietary nitrite,[17] is used as an inhibitor of color change in meat or its products, and is contained in flour, macaroni and soybean flour, being especially abundant in pickles. It should be noted that since nitrates can be reduced to nitrite in the human oral cavity by bacteria, they may also play a role. This might be environmentally important, given that nitrates are present in foods, e.g. vegetables, in considerably higher quantities than nitrites.[17]

For the present investigation of target organs with combined administration of carcinogen precursors, an established medium-term multi-organ carcinogenesis bioassay was applied.[18–20] This approach has clear benefits for examination of carcinogenic effects of chemicals in multiple organs in a single experiment within a relatively short experimental period and can detect not only carcinogenic, but also promoting action.[21–23] Hence, it is useful for elucidation of both carcinogenic and promoting effects of combined administration of compounds (MU or Mor and $NaNO_2$) in multiple organs. Furthermore, based on the proven good agreement between multi-organ carcinogenesis model data and long-term experimental results for carcinogenicity, preneoplastic lesions can be used as the end-point.[24] By using this assay, we examined the target organs of combined treatment with MNU or NNM, with particular attention to the digestive system. An assessment of nitrosation yields of MNU and NNM in the stomach, as well as adduct formation, was included for comparison purposes in the present study.

MATERIALS AND METHODS

Animals A total of 167 male F344/DuCrj rats was purchased from Charles River Japan Inc., Hino, Shiga. They were housed, 5 rats per cage, in a room with a 12 h (7 : 00–19 : 00) light-dark cycle, maintained at a constant temperature of 25±1°C and relative humidity of 55±5%. The animals were observed daily, and were used after a 1-week acclimation period for the following experiments.

Chemicals DEN, MNNG, DMH and BBN were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). DHPN was from Nakalai Tesque Co. (Kyoto), and ENUR was from Nard Institute (Nishinomiya). MU (purity >97%), Mor (purity >97%) and $NaNO_2$ (purity >97%) were from Wako Pure Chemical Ind. Ltd. (Osaka).

Treatment

Experiment 1: The experimental protocol for the multi-organ carcinogenesis model employed in the present study is shown in Fig.1. One hundred and ten male 6-week-old F344 rats were divided into 8 groups of 10 or 20 animals each. To achieve wide-spectrum initiation, animals in groups 1–6 underwent the following treatments: a single i.p. administration of 100 mg/kg body wt DEN at the commencement of the experiment, a single i.g. administration of 100 mg/kg body wt MNNG on day 14, four s.c. injections of 40 mg/kg body wt DMH on days 19, 22, 25 and 28, 0.01% ENUR plus 0.05% BBN in the drinking water for the first 2 weeks and 0.1% DHPN in the drinking water for the subsequent 2 weeks. After a one-week interval without any chemical treatment, test animals were given 0.1% MU or 0.5% Mor mixed into their diet (Oriental Powdered MF, Oriental Yeast Co., Tokyo) and 0.15% $NaNO_2$ in their drinking water for 23 weeks. Groups 1–6 were administered MU plus $NaNO_2$ (group 1), Mor plus $NaNO_2$ (group 2), MU (group 3), Mor (group 4), $NaNO_2$ (group 5) or basal diet alone (group 6), respectively. The rats in groups 7 and 8 were respectively given MU plus $NaNO_2$ and Mor plus $NaNO_2$ without the initiation regimen. The total observation period of the experiment was 28 weeks. Body weights

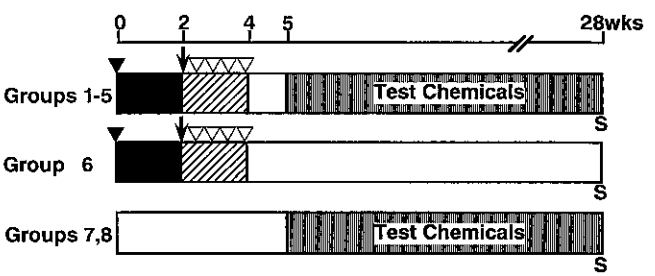

Fig. 1. Experimental protocol for the multi-organ carcinogenesis model. ▼, DEN, 100 mg/kg body wt (i.p.); ↓, MNNG, 100 mg/kg body wt (intragastrically); ▽, DMH, 40 mg/kg body wt (s.c.); ■, 0.01% ENUR+0.05% BBN in the drinking water; ▨, 0.1% DHPN in drinking water; ▥, groups 1 and 7, 0.1% MU in the diet and 0.15% $NaNO_2$ in the drinking water; groups 2 and 8, 0.5% Mor in the diet and 0.15% $NaNO_2$ in the drinking water; group 3, 0.1% MU in the diet; group 4, 0.5% Mor in the diet; group 5, 0.15% $NaNO_2$ in the drinking water; group 6, basal diet; S, animals killed.

were measured weekly up to week 12 and then every two weeks up to week 28. Total ENUR, BBN and DHPN intakes over the 4-week initiation period were measured. Levels of food and water consumption were measured every three or four weeks from weeks 5 to 28. At the end of the experiment, all survivors were killed under ether anesthesia for pathological examination.

Experiment 2: Fifty-seven male 8-week-old F344 rats were divided into 6 groups of 14 (groups 1, 2 and 6) or 5 (groups 3, 4 and 5) rats each. After overnight fasting, but with access to water, animals were given powdered basal diet (Oriental MF) containing 0.4% MU (groups 1 and 3) or 2.0% Mor (groups 2 and 4). The animals of group 5 and the control group (group 6) were given powdered basal diet only. After one hour, the food was removed and the animals of groups 1, 2 and 5 received $NaNO_2$ in distilled water (100 mg/2 ml/kg body wt, i.g.). The animals of groups 3, 4 and 6 were given distilled water only. The chemical concentrations were calculated to give equal intakes per day in experiment 1. Five rats each were killed under ether anesthesia at 1 and/or 2 h after i.g. administration for measurement of formation of *N*-nitroso compounds in all groups, and two rats each were killed at 4 and 8 h for immunohistochemical studies to detect DNA adducts in groups 1, 2 and 6 only.

Pathological examination

Experiment 1: The liver, kidneys, spleen and thymus were removed from each rat, weighed and fixed, together with the head and all other major organs except the stomach in 10% buffered formalin (pH 7.4). After adequate fixation, the nose and nasal cavities were trimmed of extraneous tissue, decalcified for approximately three days and divided into three levels for further processing as described by Young.[25] The stomach was fixed in sublimed formaldehyde made from 6% $HgCl_2$ in a mixture of 5% acetic acid and formaldehyde. All organs and tissues were embedded in paraffin and sections were stained with hematoxylin and eosin for histological examination. Liver and stomach slices were used for quantitative assessment of the development of putative preneoplastic lesions, GST-P-positive foci in the liver and PAPG in the glandular stomach, after immunohistochemical staining.[26, 27]

Quantitative assessment of preneoplastic lesions

Experiment 1: We visualized GST-P-positive foci in the liver and PAPG in the glandular stomach immunohistochemically using established procedures.[28, 29] The location of GST-P in the liver and Pg1 in the pyloric mucosa was visualized by the avidin-biotin complex immunohistochemical method with anti-rat GST-P and anti-rat Pg1 antibody, respectively.[30] The numbers and areas of GST-P-positive foci in the liver sections, as well as the areas examined, and the length of pyloric mucosa in each histological slide assessed for quantitative analysis of PAPG were measured with the aid of a color video image processor (VIP-21 C; Olympus-Ikegami Tsushin Co., Tokyo). The length of the basement membrane in the forestomach sections was also measured to allow quantitative analysis of PN hyperplasia.[31]

Measurement of formation of *N*-nitroso compounds in the stomach

Experiment 2: To demonstrate formation of *N*-nitroso compounds in the stomach, the rats were killed under ether anesthesia at 1 and 2 h after i.g. administration of $NaNO_2$ or distilled water, and their stomachs were removed and opened. The contents were pre-cooled on ice and processed according to Mirvish's procedure as follows.[32] The material was weighed, an equal amount of water was mixed in, and the pH determined. An equal weight of 0.2 *M* citrate buffer (pH 4) was then added to stop further nitrosation. For measurement of MNU, 0.2 *N* perchloric acid solution (8 ml) and *n*-hexane (5 ml) were added to samples of about 2 g, and after shaking for 5 min, and centrifugation (2000 r.p.m., 5 min), the aqueous layer was filtered through a membrane filter. The concentration of MNU in the filtrate was determined by high-performance liquid chromatography (HPLC) (pump, Shimadzu LC-10AS; detector, Shimadzu SPD-10AV). For measurement of NNM, Celite (20 g) and 1 *N* sulfuric acid containing 1% ammonium sulfamate (15 ml) were added to samples of about 3 g, and mixed. The mixture was introduced into a glass column containing 25 g of anhydrous sodium sulfate and then a further 25 g of this substance was added. Dichloromethane (200 ml) was used to elute NNM, and the eluate was concentrated to 2–100 ml by means of an evaporator at 20°C. The concentration of NNM was evaluated by the use of a gas chromatograph equipped with a thermal energy analyzer (GC-TEA) (GC part, Shimadzu GC-17A; TEA part, Thermedics Model-610). The absolute content of *N*-nitroso compounds in each stomach was calculated from the concentrations.

Detection of DNA adduct formation by immunohistochemical staining

Experiment 2: The rats were killed under ether anesthesia at 4 or 8 h after i.g. administration of $NaNO_2$. Their livers, forestomachs, esophagus and kidneys were removed, and tissue slices were fixed with acetone for 2 weeks and then embedded in paraffin. The preparation and specificity of the monoclonal O^6-medG antibodies and polyclonal N7-medG antibodies used were described previously.[33, 34] After deparaffinization, the sections were sequentially treated with 0.05 *N* sodium hydroxide in 40% ethanol for 5 min, 5% acetic acid in 40% ethanol for 15 s and 1% non-fat milk in phosphate-buffered saline for 1 h, and then exposed to primary antibodies against O^6-medG (1 : 1250) and N7-medG (1 : 2500) at 4°C over-

night.[35] After that, the avidin-biotin complex method for demonstration of binding was carried out as detailed above.

Data evaluation Statistical analysis of histopathological lesion incidences was conducted using Fisher's exact probability test. Other data were evaluated using Student's *t* test or by analysis of variance (ANOVA) and Duncan's method.

RESULTS

Survival, body weights and relative organ weights

Experiment 1: In the initiated groups (groups 1–6), twelve animals were found dead or were killed upon becoming moribund during the experiment. One animal of group 1 died at week 5 due to the toxicity of the initiation treatments. Two animals of group 1, one animal of group 3 and one animal of group 6 died with adenocarcinomas of the duodenum during weeks 26–28. Two animals of group 1 and two animals of group 6 died during weeks 18–20 for no ascertainable reason, and with no tumors on macroscopic observation. Single animals of groups 1, 3 and 6 which died during weeks 20–25 could not be examined because of autolysis. The four animals that died because of tumor development were included in the effective numbers and the others were excluded. The total intakes of BBN, ENUR and DHPN were similar in the initiated groups. Values for food and water consumption were also similar among the groups (data not shown). Final body and relative organ weights of the main organs are shown in Table I. During the initiation period, body weight gain in the initiated groups was less than that in non-treated rats. Final body weights of the combination groups (groups 1 and 2) were significantly

Table I. Final Body and Relative Organ Weights (Experiment 1)

Group	Init.[a]	Chemical	No. of rats	Final body wt (g)	Relative liver wt (%)	Relative kidney wt (%)	Relative spleen wt (%)	Relative thymus wt (%)
1	+	MU+$NaNO_2$	14	193±29[b, c]	2.6±0.2	0.82±0.08[c]	0.25±0.02	0.053±0.043
2	+	Mor+$NaNO_2$	20	262±20[c]	2.9±0.2[c]	0.69±0.03[c]	0.28±0.03	0.042±0.015
3	+	MU	8	310±25	2.6±0.2	0.59±0.04	0.26±0.04	0.036±0.010
4	+	Mor	10	292±20	2.6±0.2	0.64±0.03	0.27±0.04	0.040±0.007
5	+	$NaNO_2$	10	316±13	2.3±0.1	0.61±0.03	0.25±0.02	0.040±0.010
6	+	—	16	317±19	2.5±0.2	0.59±0.05	0.26±0.05	0.031±0.005
7	—	MU+$NaNO_2$	10	239±13	2.5±0.6	0.74±0.05	0.35±0.51	0.033±0.020
8	—	Mor+$NaNO_2$	10	309±11	2.7±0.1	0.65±0.03	0.21±0.01	0.043±0.011

a) Treatments for multi-organ initiation.
b) Mean±SD.
c) Significantly different from group 6 at $P<0.05$ (ANOVA→Duncan's method).

Table II. Incidences of Preneoplastic and Neoplastic Lesions in the Esophagus, Forestomach and Liver (Experiment 1)

Organ and lesions	Group	1	2	3	4	5	6	7	8
	Initiation	+	+	+	+	+	+	−	−
	Chemical	MU+$NaNO_2$	Mor+$NaNO_2$	MU	Mor	$NaNO_2$	−	MU+$NaNO_2$	Mor+$NaNO_2$
	No. of rats	16	20	9	10	10	17	10	10
Esophagus									
Papilloma		1 (6)[a]	0	0	0	0	0	0	0
PN hyperplasia		7 (44)[b]	0	0	0	0	0	0	0
Forestomach									
SCC		3 (19)	2 (10)	0	1 (10)	1 (10)	1 (6)	0	0
Papilloma		10 (63)	12 (60)	0	5 (50)	5 (50)	6 (35)	0	0
SCC or Papilloma		11 (69)	13 (65)	0	5 (50)	5 (50)	7 (41)	0	0
PN hyperplasia		16 (100)	16 (80)	5 (56)	6 (60)	10 (100)	13 (76)	0	0
Liver									
Hyperplastic nodule		0	2 (10)	0	0	0	0	0	0

PN, papillary or nodular; SCC,squamous cell carcinoma.
a) Number of animals bearing lesions. The numbers in parentheses are percentage incidences.
b) Significantly different from other groups at $P<0.05$ (Fisher's exact probability test).

Table III. Incidences of Preneoplastic and Neoplastic Lesions in Other Organs (Experiment 1)

Organ and lesions	Group	1	2	3	4	5	6	7	8
	Initiation	+	+	+	+	+	+	−	−
	Chemical	MU+$NaNO_2$	Mor+$NaNO_2$	MU	Mor	$NaNO_2$	−	MU+$NaNO_2$	Mor+$NaNO_2$
	No. of rats	16	20	9	10	10	17	10	10
Duodenum									
Adenocarcinoma		6 (38)[a]	3 (15)	5 (56)	1 (10)	1 (10)	4 (24)	0	0
Adenoma		0	0	0	0	0	0	0	0
Lymphoma		0	0	0	0	0	0	1 (10)	0
Large intestine									
Adenocarcinoma		3 (19)	4 (20)	0	2 (20)	0	4 (24)	0	0
Adenoma		1 (6)	0	0	0	0	0	0	0
Kidney									
Adenocarcinoma		1 (6)	0	0	0	0	1 (6)	0	0
Adenoma		0	1 (5)	0	1 (10)	1 (10)	2 (12)	0	0
Nephroblastoma		0	2 (10)	0	2 (20)	1 (10)	2 (12)	0	0
TCC		3 (19)	0	0	0	0	2 (12)	0	0
Lung									
Adenocarcinoma		3 (19)	4 (20)	0	2 (20)	1 (10)	2 (12)	0	0
Adenoma		7 (44)	3 (15)	3 (33)	2 (20)	2 (20)	2 (12)	0	0
SCC		0	1 (5)	0	0	0	0	0	0
Urinary bladder									
TCC		1 (6)	0	0	0	0	0	0	0
Papilloma		0	0	0	0	0	2 (12)	0	0
PN hyperplasia		2 (13)	4 (20)	2 (22)	2 (20)	3 (30)	2 (12)	0	0
Thyroid gland									
Adenocarcinoma		0	0	0	0	0	0	1 (10)	0
Adenoma		1 (6)	0	0	1 (10)	0	0	0	0
Medullary carcinoma		0	1 (5)	0	0	0	0	0	0
C cell adenoma		0	0	0	0	0	1 (6)	0	0
Nasal cavity									
Carcinoma		0	0	0	1 (10)	0	0	0	0
Papilloma		0	3 (15)	0	0	0	1 (6)	0	0
PN hyperplasia		1 (6)	7 (35)	0	3 (30)	1 (10)	4 (24)	0	0
Fibroma		0	0	0	0	0	0	1 (10)	0
Spleen									
Malignant lymphoma		0	0	0	0	0	0	1 (10)	0

TCC, transitional cell carcinoma; SCC, squamous cell carcinoma; PN, papillary or nodular.
a) Number of animals bearing lesions. The numbers in parentheses are percentage incidences.

reduced as compared with the control group (group 6), especially in the MU plus $NaNO_2$ case. The relative liver weights in group 2 and the relative kidney weights in groups 1 and 2 were significantly higher than those in the control group (group 6).

Incidences of neoplastic and preneoplastic lesions

Experiment 1: Incidences of preneoplastic and neoplastic lesions in various organs are summarized in Tables II and III. Papillomas and PN hyperplasias in the esophagus were only induced with statistical significance in group 1. In the forestomach, incidences of tumors and PN hyperplasias of groups 1 and 2 showed a tendency for elevation as compared to the control group (group 6). Similarly, $NaNO_2$, which is known to promote forestomach carcinogenesis,[36,37] caused what appeared to be a slight increase in hyperplastic hepatic nodules only when given together with Mor. In the initiated groups (groups 1–6), neoplastic and preneoplastic lesions were observed in several other organs or tissues, such as the duodenum, large intestine, kidney, lung, urinary bladder, thyroid gland, nasal cavity and spleen, but without remarkable inter-group differences. In the non-initiated groups (groups 7 and 8) the few neoplastic changes observed in the thyroid, nasal cavity and spleen were not significant.

Quantitative assessment of preneoplastic lesions

Experiment 1: Fig. 2 and Table IV summarize the data for GST-P-positive foci in the liver, PN hyperplasia in the forestomach[38] and PAPG numbers in the glandular stomach. The numbers and areas of GST-P-positive foci were significantly increased in group 1 and even more

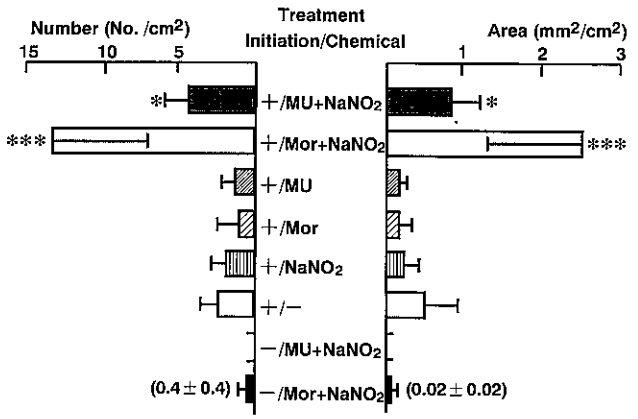

Fig. 2. Quantitative data for numbers and areas of GST-P-positive foci in the livers of rats in experiment 1. Significantly different from group 6 at $*P<0.05$, $***P<0.001$ (Student's t test).

markedly in group 2 (Mor plus $NaNO_2$) compared to group 6. The combination of Mor plus $NaNO_2$ induced GST-P-positive foci even in the non-initiated group (group 8). The numbers of PN hyperplasias per basement membrane length in the forestomach in group 1 were significantly higher than in groups 3, 5 and 6. There were no significant intergroup differences in the numbers of PAPGs in the glandular stomachs. In the non-initiated groups (groups 7 and 8), no induction of PN hyperplasia in the forestomach or PAPG in the glandular stomach was apparent.

Formation of *N*-nitroso compounds in the stomach

Experiment 2: The results of measurement of formation of *N*-nitroso compounds in the stomach are summarized in Table V. The mean MNU yields in group 1 (MU plus $NaNO_2$) were 6050 and 7700 μg at 1 and 2 h, respectively, after the precursor administration. The volumes of MNU were 36.9 and 45.9% of the MU mixed in the basal

Table IV. Quantitative Data for Preneoplastic Lesions in the Stomachs of Initiated Rats (Experiment 1)

Group	Chemical	No. of rats	Forestomach: PN hyperplasia (No./10 cm BM)	Glandular stomach: PAPG (No./10 cm PM)
1	MU+$NaNO_2$	14	10.2±5.4[a, b]	18.8±7.1
2	Mor+$NaNO_2$	20	4.1±3.2	18.2±9.5
3	MU	8	3.0±3.2	20.5±8.8
4	Mor	10	2.3±2.1	16.3±9.5
5	$NaNO_2$	10	4.1±1.3	16.3±8.0
6	—	16	5.7±4.8	11.3±5.5

PN, papillary or nodular; PAPG,pepsinogen 1 altered pyloric glands.
BM, basement membrane; PM,pyloric mucosa.
a) Mean±SD.
b) Significantly different from groups 3, 5 and 6 at $P<0.05$ (ANOVA→Duncan's method).

Table V. Formation of *N*-Nitroso Compounds in the Stomach (Experiment 2)

Group	Chemical	No. of rats	Time after treatment	Stomach content (g)	*N*-Methyl-*N*-nitrosourea (μg)	*N*-Nitrosomorpholine (μg)
1	MU+$NaNO_2$	5	1 h	4.1±1.1[a]	6050±1794	NE
		5	2 h	4.2±0.7	7700±1548	NE
2	Mor+$NaNO_2$	5	1 h	3.2±0.7	NE	5300±1208
		5	2 h	3.0±1.3	NE	6720±2802
3	MU	5	2 h	3.1±0.8	<100	NE
4	Mor	5	2 h	3.1±1.7	NE	<0.3
5	$NaNO_2$	5	2 h	6.3±1.4	<200	2.6±2.2
6	—	5	1 h	4.5±0.8	<100	1.1±1.1
		5	2 h	4.3±1.2	<100	0.9±0.8

NE, not examined.
a) Mean±SD.

Fig. 3. Immunohistochemical staining of N7-medG in the forestomach of a rat treated with MU plus $NaNO_2$ in experiment 2. In this figure, a single cell in stratified squamous epithelium is positive for anti N7-medG antibody binding (arrow). (Bar=50 μm)

diet. The mean NNM yields in group 2 (Mor plus $NaNO_2$) were 5300 and 6720 μg at 1 and 2 h respectively, after the administration. The formed volumes of NNM were 5.9 and 7.8% of the administered Mor. Levels of *N*-nitroso compounds detected after administration of MU, Mor or $NaNO_2$ alone (groups 3, 4 and 5) were similar to the group 6 values.

Immunohistochemical staining of DNA adducts

Experiment 2: In group 1, but not group 2, N7-medG-positive nuclei were found in the squamous epithelium of the forestomach at 8 h after the combined precursor administration, but were few in number (Fig. 3), whereas the forestomach epithelium of the control group was negative. On the other hand, at 4 h after administration, N7-medG-positive cells were not detected in the forestomach. However in the esophagus, liver and kidney of groups 1 and 2, N7-medG-positive cells were not detected even at the 8 h time point. No O^6-medG-positive cells were detected in the target organs in any group.

DISCUSSION

The combination of MU plus $NaNO_2$ was earlier reported to result in neurogenic and lymphoid neoplasms,[10] or lung adenomas in Swiss mice,[7,8] despite the wide-spectrum carcinogenicity known to be exerted by MNU. In the present study, however, the combination of MU plus $NaNO_2$ increased development of lesions in the digestive system, such as the esophagus, forestomach and liver, in line with the target organs of MNU. The combination of Mor plus $NaNO_2$ was also demonstrated to cause liver carcinogenicity, in accordance with previous studies.[8,9] Furthermore, *N*-nitroso compound formation in the stomach was confirmed.

The fact that the final body weights of rats in the combination groups were decreased in experiment 1 could be explained by formation of *N*-nitroso compounds and resultant toxicity, especially with the MU plus $NaNO_2$ combination. The significant increase in relative liver weights in group 2 is also consistent with a growth effect underlying promotion of GST-P-positive foci.

The induction of esophageal tumors and preneoplasias in group 1 given MU plus $NaNO_2$ can be concluded to be a treatment effect since no such lesions were observed in the other groups. This is of interest, given the reported DNA adduct formation with MNU in the rat esophagus.[39] While the apparent increase in neoplastic and preneoplastic lesions in the forestomach observed in group 1 might have been due partly to $NaNO_2$ itself, a significant influence of endogenously formed MNU was noted.

The development of GST-P foci in the livers of animals receiving the combination of Mor plus $NaNO_2$, with or without prior initiation, is in line with NNM hepatocarcinogenicity. The results for group 1 are similarly in agreement with a weak promotion action of MNU.[40,41]

Mirvish *et al.* found that the MNU yield in the rat stomach was 27% of the MU administered as a solution (21.5 mg) together with sodium nitrite (10 mg).[42] Subsequently they demonstrated that the yield was only 0.46% of the MU when MU was given in the food (100 mg MU/kg in food and 4 g $NaNO_2$/kg in drinking water).[43] In our case, the value was 36.9% of the MU 1 h after administration and 45.8% after 2 h. Thus, the amounts of MNU formed were extremely high in this experiment. Although ordinarily the formation of *N*-nitroso compounds from secondary amines is low, the NNM yield, at 5.9% of the Mor after 1 h and 7.8% of the Mor after 2 h, was also relatively high.

The finding of N7-medG-positive nuclei in the forestomach of rats given MU plus $NaNO_2$ was as expected given the nature of MNU as a direct carcinogen that methylates DNA without undergoing prior metabolism.[44] However, the number of N7-medG-positive cells was not large in this study. The lack of O^6-medG-positive cells in the forestomach is probably due to the lower initial formation and more rapid repair of these adducts compared with N7-medG, resulting in a level of adduct below the detection limit of the immunohistochemistry (approximately 1 μmol of adduct per parent nucleoside). The absence of positive staining for both adducts in the other organs argues for a lower level of exposure than in the forestomach. O'Connor *et al.* also showed that cells with nuclei containing O^6-medG after MNNG administration were heterogeneously distributed in the digestive tract.[45] Little is known about the mechanism of carcinogenesis by NNM but our data suggest that methylation of

DNA does not occur at a high level in this context. It should be borne in mind, however, that Hecht *et al.* have demonstrated formation of cyclic DNA adducts with this compound.[46, 47]

The risk associated with the intake of *N*-nitroso precursors is a function of three variables: 1) the amounts of precursor and nitrite ingested, 2) the rate of *in vivo* nitrosation and 3) the carcinogenic potency of the resulting *N*-nitroso compounds. The ureas are potentially more important than secondary amines as risk factors because of their ready nitrosation.[2] Generally secondary amines such as Mor are low-risk factors because of low nitrosation. However, unexpectedly, the formation of NNM from Mor was equal to that of MNU. The combination of Mor plus $NaNO_2$ thus also carries high risk.

In our study, the concentration of $NaNO_2$ applied was 0.15% in water. $NaNO_2$ is included in hams and sausages at a level of about 15–80 ppm, and also in liquids for preserving pickles (about 100 ppm). Nitrates are abundant in pickled and also non-pickled vegetables, particularly in Japan (1500–8000 ppm).[17, 48] In Europe and North America the average daily intake of $NaNO_2$ is in the region of around 4 mg/person. According to Stephany and Shuller, ingestion of nitrates is much higher (100 mg/person/day), and about 6% is reduced endogenously to nitrite.[48] However, the rate-limiting factor for nitrosation is the concentration of amines or amides. Mirvish *et al.* have reported finding 25 ppm of MU in both Japanese fish products and in fried bacon.[13] The 0.1% MU given in the diet in this study was a 40 times higher concentration than that in human foodstuffs. Moreover, the variety of the human diet means that the dose of MU ingested would normally be very low. However, Rickard *et al.* reported that about 30% nitrosation with MU plus $NaNO_2$ occurred in the guinea pig as opposed to 0.5% in the rat (1/60 of that in guinea pig).[49] The difference was attributed to the pH of the gastric contents, with that in the rat, ranging from 3 to 5, contrasting strongly with the pH of 1 or 2 prevailing in the guinea pig.[49, 50] Since the pH of the human stomach is also in the range of 1–2, it is likely that nitrosation in human stomach is also many times higher than in rats.[51] We therefore can not ignore the risk of carcinogenicity from combinations of precursor compounds in our diet. Further study of this area and the question of species variation is clearly warranted.

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