

Induction of Glandular Stomach Cancers in *Helicobacter pylori*-sensitive Mongolian Gerbils Treated with *N*-Methyl-*N*-nitrosourea and *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine in Drinking Water

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An animal model of stomach carcinogenesis was established using Mongolian gerbils with *N*-methyl-*N*-nitrosourea (MNU) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as the carcinogens. In addition, the sensitivity of these gerbils to *Helicobacter pylori* (*H. pylori*) was confirmed. One hundred and sixty specific pathogen-free male MGS/Sea animals, 7 weeks old, were treated with MNU in the drinking water (30 ppm for alternate weeks to give 10 weeks exposure, or 10 ppm or 3 ppm for 20 weeks continuous exposure), or given MNNG in the drinking water at 400 ppm or 200 ppm for 20 weeks, or orally inoculated with ATCC43504 *H. pylori* (1.7×10^8 CFUs/animal). Adenocarcinomas in the glandular stomach were found in 2 out of 12 effective animals (2/12) treated with 30 ppm MNU at week 20, although all were dead or moribund by week 30 due to MNU toxicity. At week 50, the incidences of gastric adenocarcinomas in groups treated with 10 ppm MNU, 3 ppm MNU, 400 ppm MNNG, and 200 ppm MNNG were 2/21 (9.5%), 1/23 (4.3%), 7/11 (63.6%), and 1/10 (10.0%). The lesions were generally well differentiated, although poorly differentiated adenocarcinoma was also found in a single gerbil in each of the 10 ppm MNU and 400 ppm MNNG groups. In control animals no tumors were found. In the infection study, the animals were killed at week 20, and *H. pylori* was detected in all cases, causing multiple erosions with marked inflammatory cell infiltration in the lamina propria and submucosa, and frequent formation of lymphoid follicles. Thus, MNU and MNNG in the drinking water induced neoplastic lesions in the glandular stomach epithelium of *H. pylori*-sensitive gerbils.

Key words: Glandular stomach cancer — *Helicobacter pylori* — Mongolian gerbil — *N*-methyl-*N*-nitrosourea — *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

Helicobacter pylori (*H. pylori*) has been linked to chronic atrophic gastritis, a precursor condition for gastric carcinoma.¹⁻³ Based on epidemiological findings, *H. pylori* was defined as a “definite biological carcinogen” by WHO/IARC in 1994.⁴ However, there is also a report of an apparent lack of association between *H. pylori* infection and risk of gastric cancer.⁵ Many animals infected with human *H. pylori* have already been studied to determine the pathogenetic background,⁶⁻¹² but none of the models studied mimics human *H. pylori* infection and subsequent pathology. Recently, however, a Mongolian gerbil model of human *H. pylori* infection, with the bacteria detectable throughout a 12-month study period, was described.¹³ In this model, gastric ulcers and intestinal metaplasia were induced, although no neoplastic lesions were found. For analysis of the role of *H. pylori* in gastric carcinogenesis, therefore, establishment of an experimental model of gastric carcinogenesis in the Mongolian gerbil is very important. In the present study, the sensitivity of this animal to *N*-methyl-*N*-nitrosourea (MNU) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) gastric

carcinogenicity was investigated. In addition, the susceptibility of the stomach to human *H. pylori* was confirmed.

One hundred and sixty specific pathogen-free male gerbils (*Meriones unguiculatus*) (MGS/Sea) (Seac Yoshitomi, Ltd., Fukuoka), 6 weeks old, were housed in plastic cages with hard wood chips in an air-conditioned biohazard room for infection with a 12 h light-12 h dark cycle. They were given food (Oriental NMF, Oriental Yeast Co., Tokyo) and water *ad libitum*. After one week, the animals were divided into three groups for Experiments I, II and III. MNNG (Tokyo Kasei Kogyo Co., Ltd., Tokyo) and MNU (Sigma Chemical Co., St Louis, MO) were each dissolved in distilled water (solutions were freshly prepared three times per week) for administration in light-shielded bottles as drinking water *ad libitum*. *H. pylori* (ATCC43504, American Type Culture Collection, Rockville, MD) was inoculated on brucella agar plates (Remel, Lenexa, KS) containing 7% heat-inactivated fetal bovine serum and incubated at 37°C under microaerobic conditions using Anaero Pack Campylo (Mitsubishi Gas Chemical Co., Inc., Tokyo) at high humidity. Two days later,

the bacteria grown on the plates were collected with inoculating loops, dissolved in 0.5 ml of brucella broth (Remel), introduced into brucella broth supplemented with 7% heat-inactivated fetal bovine serum in tissue culture flasks with Vent caps, and incubated at 37°C under microaerobic conditions at high humidity for 24 h without shaking. The broth cultures were checked by phase contrast microscope for shape and mobility of *H. pylori*. Samples containing 2.1×10^8 colony-forming units (0.8 ml) per milliliter were used as the inoculum, delivered via an oral catheter.

The animals were treated as follows (Fig. 1): Experiment I: Ninety gerbils were divided into 3 groups. They were given MNU in their drinking water at a concentration of 30 ppm for alternate weeks to give 10 weeks intermittent exposure (group 1), or at a concentration of 10 ppm (group 2) or 3 ppm (group 3) for 20 weeks continuously, and then given tap water. Experiment II: Forty gerbils were divided into 2 groups given MNNG in their drinking water at a concentration of 400 ppm (group 1) or 200 ppm (group 2) for 20 weeks, followed by tap water. Experiment III: *H. pylori* (1.7×10^8 CFUs/animal) was given by intragastric intubation (i.g.) to 10 gerbils after they had been subjected to starvation for 24 h. Four hours after inoculation, they were allowed free access to water and fed again, and all animals were killed at week 20. As non-treated controls for experiments I, II and III, 20 gerbils were used. The experimental animals were initially weighed every week and then every two to three weeks after week 20. Necropsies were performed on all animals which died or were killed upon becoming moribund. In experiments I and II, ten to twelve (experiment I) and six or seven (experiment II) animals were killed at week 20

and all surviving animals were killed and autopsied at the end of the 30th experimental week (group 1 in experiment I) or at the end of the 50th experimental week (the others). Animals that survived for 20 weeks (group 1 in experiment I) or 30 weeks (others), when the first tumors appeared, were included in the effective numbers for analysis of the incidence of tumors. Survival curves of gerbils were calculated without including gerbils killed on schedule at week 20. For detection of *H. pylori* infection, samples of about 30 mm² of stomach mucosa from the greater curvature, containing both fundic and pyloric glands, were homogenized with one ml of brucella broth. Aliquots of 0.1 ml were inoculated on segregating agar plates for *H. pylori* (Eiken Chemical Co., Tokyo) and incubated at 37°C under microaerobic conditions using Anaero Pack Campylo (Mitsubishi Gas Chemical Co., Inc.) at high humidity for six days. The excised stomachs were fixed in sublimed formaldehyde and cut into about 6 strips for embedding in paraffin. Other tissues were carefully checked under the naked eye. Tumors and related lesions were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (HE), and alcian blue-PAS, as well as by the paradoxical concanavalin A (Con A) method¹⁴⁾ and by immunohistochemistry for *H. pylori* (anti-*H. pylori* serum, Dako, Denmark). Neoplastic lesions of the glandular stomach were classified as adenomas and adenocarcinomas.¹⁵⁾ Adenomas consisted of excessive glandular proliferation with scanty cellular atypia. Adenocarcinomas of the glandular stomach were classified into well differentiated lesions characterized by tubular structures, poorly differentiated tumors characterized by little tendency to form glandular structures with severe cellular atypia, and signet

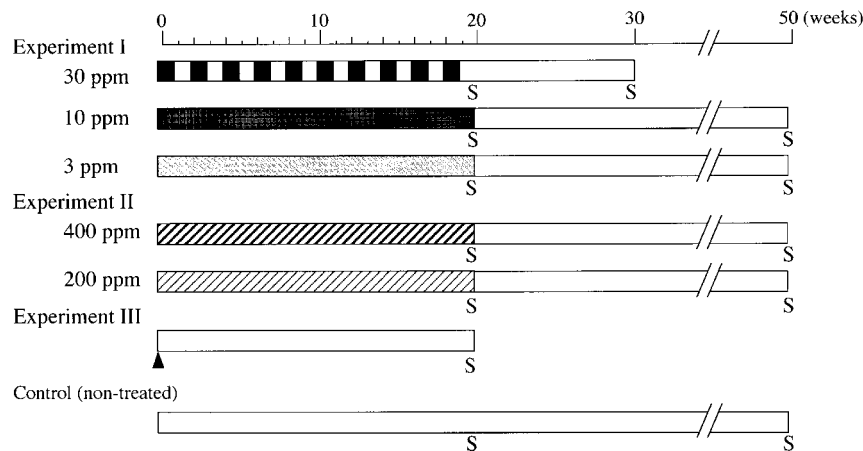


Fig. 1. Experimental design. Animals, male Mongolian gerbils 7 weeks old; S, animals were killed. ■ MNU in the drinking water, ▨ MNNG in the drinking water, ▲ *H. pylori* (1.5×10^8 CFUs) by i.g.

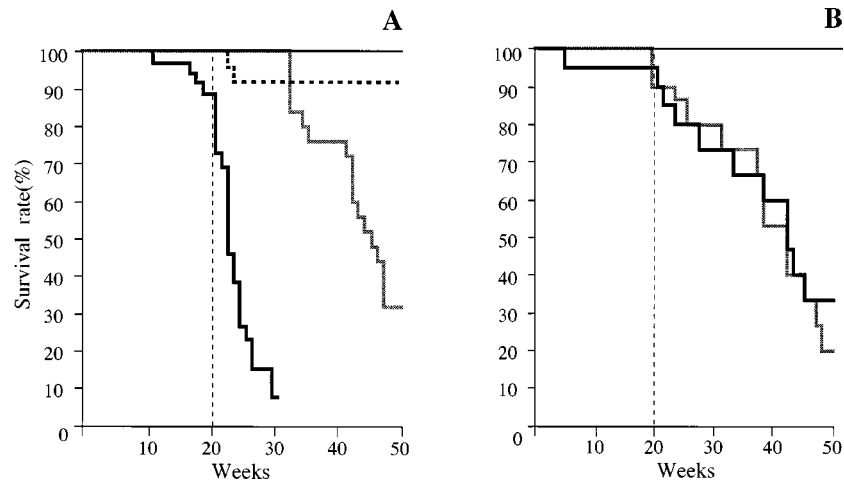


Fig. 2. Survival curves of gerbils treated with MNU (A) or MNNG (B). A, — 30 ppm, — 10 ppm, - - - 3 ppm, — control. B, — 400 ppm, — 200 ppm, — control.

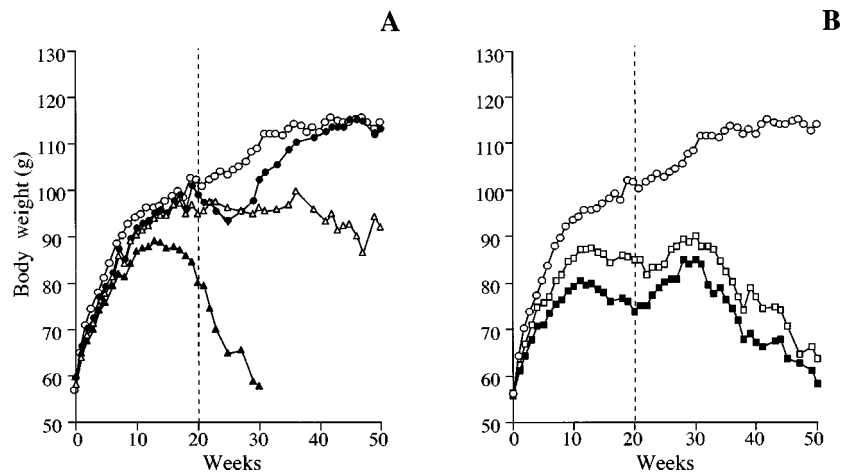


Fig. 3. Body weight curves of gerbils treated with MNU (A) or MNNG (B). A, ▲ 30 ppm, △ 10 ppm, ● 3 ppm, ○ control. B, ■ 400 ppm, □ 200 ppm, ○ control.

ring cell carcinomas characterized by isolated tumor cells containing abundant mucin.

In experiments I and II, reductions in survival and body weight were observed in gerbils treated with MNU or MNNG in a dose-dependent manner (Figs. 2 and 3). In group 1 receiving 30 ppm of MNU in experiment I, almost all the gerbils died or became moribund from weeks 20 to 30 and the 2 remaining gerbils were therefore killed at week 30. At week 20 in the groups given MNU (30 ppm) and MNNG (400 and 200 ppm), ulcers and dysplastic regenerated epithelium were found in the glandular stomach mucosa in almost all animals. However, gerbils

treated with 10 ppm or 3 ppm MNU showed minimal changes. The incidences of gastrointestinal tumors observed in each group are summarized in Tables I and II. The tumors were generally limited to the forestomach, glandular stomach and duodenum. All forestomach lesions were of squamous cell origin. Most tumors in the glandular stomach were found in the pyloric mucosa adjacent to the fundic region. At week 20, adenocarcinomas in the glandular stomach were found only in the group treated with 30 ppm MNU and no tumors were apparent in the other groups treated with carcinogens. At week 50, adenocarcinomas were found at low incidence in the 10

Table I. Incidence of Tumors in the Forestomach, Glandular Stomach and Small Intestine of Mongolian Gerbils Treated with MNU (Experiment I)

Treatments and weeks	Effective no. of gerbils	Forestomach		Glandular stomach				Small intestine	
		Squamous cell carcinoma	Adenoma	Adenocarcinoma			Sarcoma	Adenocarcinoma	
				Incidence	Histology				
Well	Poorly	Signet							
30 ppm									
20 w	12	3 (25.0)	2 (16.7)	2 (16.7)	1	1	1	0	5 [1] (41.7)
30 w	17	7 (41.2)	3 (17.6)	2 (11.8)	2	0	0	0	8 [1] (47.1)
10 ppm									
20 w	10	0	0	0	0	0	0	0	0
50 w	21	1 (4.8)	5 (23.8)	2 (9.5)	3	1	0	1 (4.8)	4 (19.0)
3 ppm									
20 w	10	0	0	0	0	0	0	0	0
50 w	23	0	8 (34.8)	1 (4.3)	1	0	0	2 (8.7)	0
Control (0 ppm)									
20 w	5	0	0	0	0	0	0	0	0
50 w	10	0	0	0	0	0	0	0	0

Well, well differentiated adenocarcinoma; Poorly, poorly differentiated adenocarcinoma; Signet, signet ring cell carcinoma. () %, [] No. of Brunner's gland adenocarcinoma.

Table II. Incidence of Tumors in the Forestomach, Glandular Stomach and Small Intestine of Mongolian Gerbils Treated with MNNG (Experiment II)

Treatments and weeks	Effective no. of gerbils	Forestomach		Glandular stomach				Small intestine
		Squamous cell carcinoma	Adenoma	Adenocarcinoma			Sarcoma	Adenocarcinoma
				Incidence	Histology			
Well	Poorly							
400 ppm								
20 w	6	2 (33.3)	0	0	0	0	0	2 (33.3)
50 w	11	5 (45.5)	4 (36.4)	7 (63.6)	6	1	4 (36.4)	7 [2] (63.6)
200 ppm								
20 w	7	3 (42.9)	0	0	0	0	0	0
50 w	10	4 (40.0)	4 (40.0)	1 (10.0)	1	0	0	4 [2] (40.0)
Control (0 ppm)								
20 w	5	0	0	0	0	0	0	0
50 w	10	0	0	0	0	0	0	0

Well, well differentiated adenocarcinoma; Poorly, poorly differentiated adenocarcinoma. () %, [] No. of Brunner's gland adenocarcinoma.

ppm MNU group and at high incidence in the 400 ppm MNNG group. Carcinomas induced by MNNG were mainly well differentiated adenocarcinomas (Fig. 4), though well differentiated and poorly differentiated (Fig. 5) adenocarcinomas and a signet ring cell carcinoma (Fig. 6) were induced in the glandular stomach at low incidences by MNU. Intestinal metaplasia was not evident in any stomach in experiments I and II. Sarcomas also developed in the glandular stomach, with 4 leiomyosarcomas in group 1 of experiment II (MNNG 400 ppm). In the small

intestine, including the duodenum, adenocarcinomas were found at relatively high incidences in the MNU and MNNG treated groups. Adenocarcinomas originating from Brunner's glands were included. No tumors were detected in the large intestine and control animals lacked any neoplastic lesions.

In experiment III, *H. pylori* was detected by culture in all gerbils previously inoculated, whereas all control animals were negative. The numbers of colonies were around 10⁵ CFUs per stomach for all inoculated gerbils.

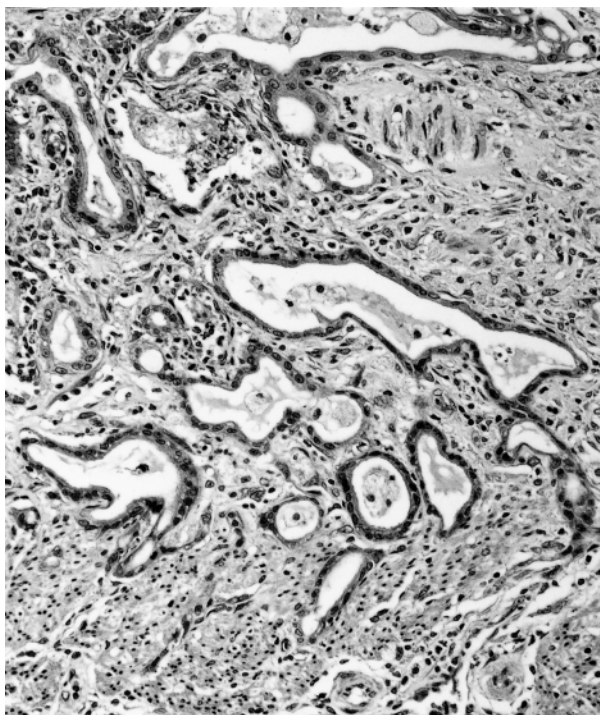


Fig. 4. A typical well differentiated glandular stomach adenocarcinoma at week 50 in an animal of experiment II treated with 400 ppm MNNG. HE, $\times 200$.

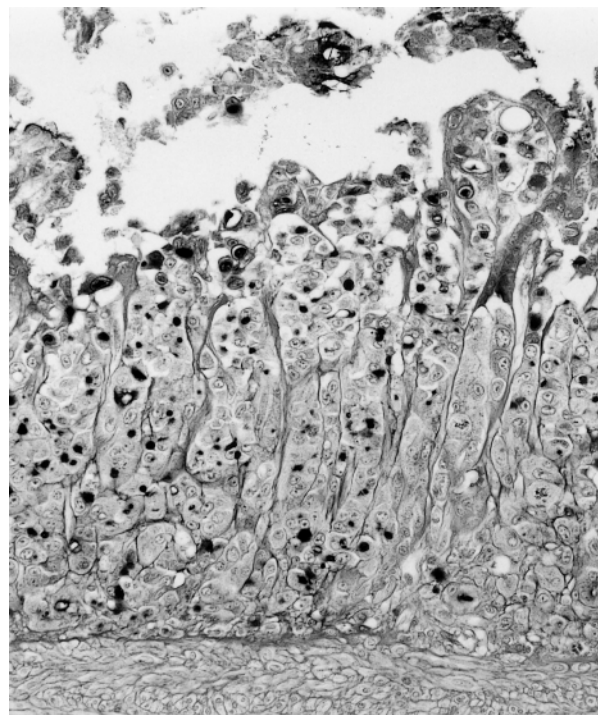


Fig. 5. A poorly differentiated glandular stomach adenocarcinoma at week 50 in an animal of experiment I treated with 10 ppm MNU. Alcian blue-PAS, $\times 200$.

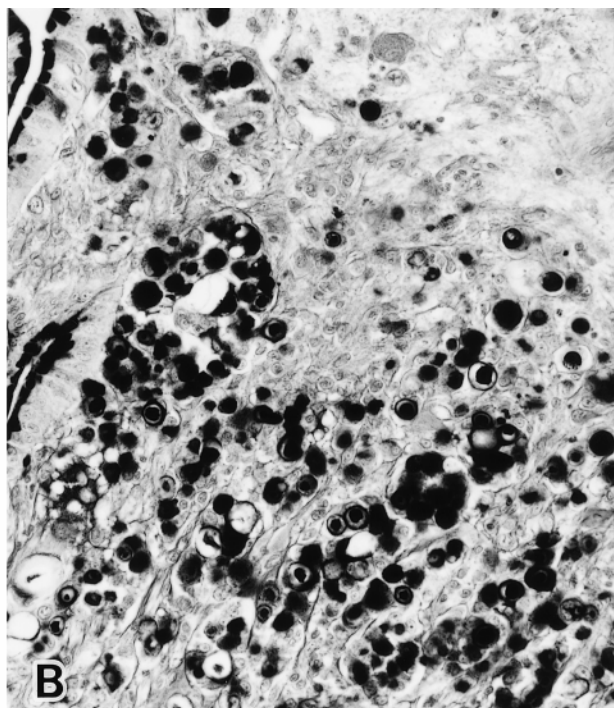
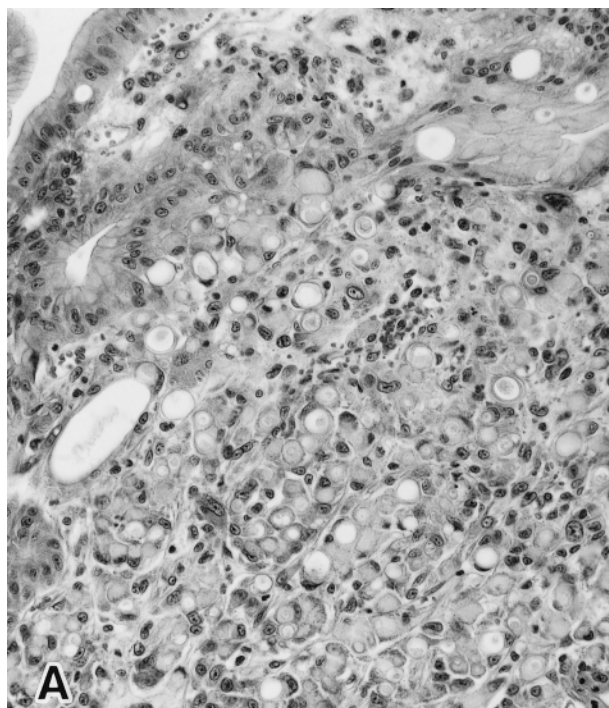


Fig. 6. A, A glandular stomach signet ring cell carcinoma at week 20 in an animal in experiment I treated with 30 ppm MNU. HE, $\times 200$. B, Serial section of the same specimen as in Fig. 6A. Alcian blue-PAS, $\times 200$.

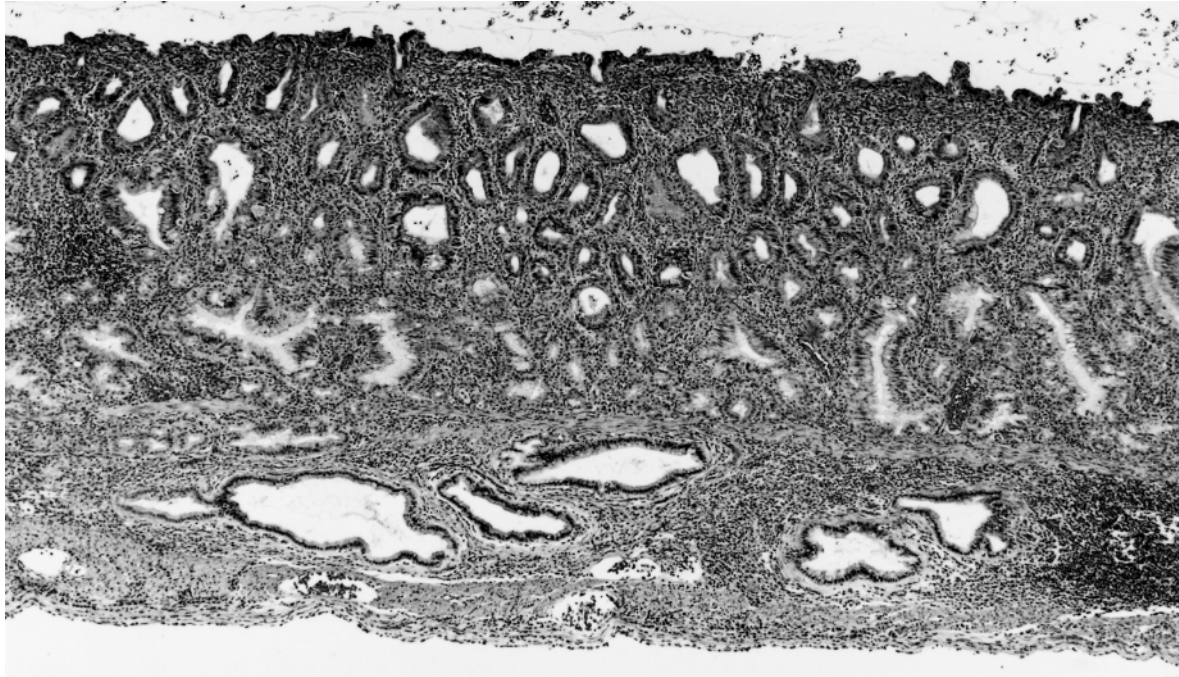


Fig. 7. Severe gastritis at week 20 with marked infiltration of inflammatory cells and glands in the submucosa in an animal of experiment III infected with *H. pylori*. HE, $\times 40$.

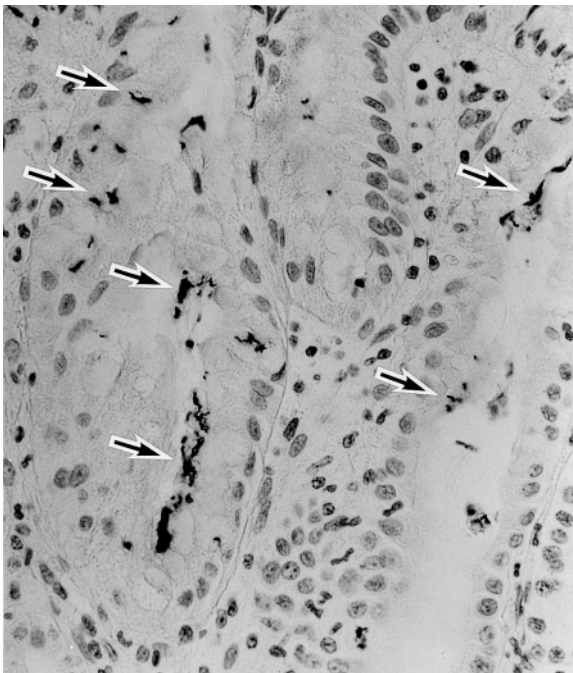


Fig. 8. Bacteria (arrows) are evident in the gastric pits and surface mucus layer at week 20 in an animal of experiment III infected with *H. pylori*. Immunohistochemical staining of *H. pylori*, $\times 400$.

Macroscopically, the glandular stomachs of the affected animals were edematous, with hemorrhagic spots and erosions being found. Histologically, the glandular gastric epithelium showed hyperplastic changes with variable degrees of cystic glandular dilatation and multifocal erosion. The bases of the glands had broken through the muscularis mucosae multifocally and multiple cystic glands were present in the submucosa. There was marked infiltration, predominantly of lymphocytes and some macrophages, as well as neutrophils, in the lamina propria and submucosa, with frequent formation of lymphoid follicles (Fig. 7). Immunohistochemistry also demonstrated the existence of *H. pylori* in all inoculated gerbils (Fig. 8). No neoplastic or intestinal metaplastic changes were found at week 20. Moreover, there was no change in the distribution of class III mucins, so there was no apparent phenotypic alteration in the mucous membrane. In the control, there were no pathological changes of note.

The present investigation clearly demonstrated that MNU and MNNG can induce neoplasms in the glandular stomach of gerbils, with dose-dependent effects on body weight, survival time and tumor development. Intermittent administration of 30 ppm MNU for 10 one-week periods induced well and poorly differentiated adenocarcinomas and a signet ring cell carcinoma within 20 weeks. The histological types of adenocarcinomas induced by MNU

varied considerably, as demonstrated earlier for counterpart lesions 7in mice.¹⁵⁻¹⁷⁾ However, since toxic effects were pronounced with the high dose of MNU, lower concentrations are required to ensure longer survival. In contrast, gerbils proved resistant to MNNG, so that relatively high dosages were tolerated. The incidences of glandular stomach adenocarcinomas in the groups given 400 and 200 ppm MNNG were dose-dependent, at 7/11 and 1/10. Almost all the tumors were well differentiated, as observed in rats treated with MNU¹⁸⁾ and MNNG.^{19,20)}

Intestinal metaplasia in humans has been considered to be a preneoplastic change²¹⁻²³⁾ for well differentiated adenocarcinomas. However, well-differentiated adenocarcinomas contain gastric-type cancer cells with class III mucins, pepsinogens or mucins specific to gastric surface mucous cells, suggesting their origin to be gastric epithelium. Independence of gastric phenotypic expression of stomach cancer cells and surrounding intestinal metaplastic mucosa has also been reported.²⁴⁾ As no intestinal metaplasia was noted in any of the stomachs in the present experiment, no relationship between intestinal metaplasia and MNU- or MNNG-induced glandular stomach cancers in gerbils was found. The data in this work are also consistent with the conclusion from our previous studies²⁵⁻²⁷⁾ that intestinal metaplasia is not a preneoplastic change of any major relevance to gastric neoplasia.

Twenty weeks after a single inoculation of *H. pylori*, the bacteria were detectable, along with gastritis and erosion. This confirms that Mongolian gerbils resemble man

in their susceptibility and response to infection. It has already been reported that pathological changes progress for up to 12 months and that intestinal metaplasia may also be induced in the glandular stomach of these gerbils in response to *H. pylori*.¹³⁾ Therefore the gerbil model appears admirably suited to investigating the role of *H. pylori* in human gastric disorders, including the increased risk of gastric adenocarcinoma.¹⁻⁴⁾ It is clear, however, that bacterial infection alone cannot explain the pathogenesis of gastric carcinoma. *H. pylori* infection is extraordinarily common, and in some developing nations it affects almost all adults.²⁸⁾ Only a very small percentage of infected persons will develop stomach neoplasms. So there must be other critical factors.

In conclusion, MNU and MNNG can induce glandular stomach cancer in Mongolian gerbils so this animal, which is highly susceptible to *H. pylori* infection, may afford a good model for elucidation of the interaction between bacterial infection and carcinogens in the induction of stomach cancer.

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REFERENCES

- 1) Warren, J. R. and Marshall, B. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet*, **i**, 1273-1275 (1983).
- 2) Parsonnet, J., Friedman, G. D., Vandersteen, D. P., Chang, Y., Vogelman, J. H., Orentreich, N. and Sibley, R. K. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Engl. J. Med.*, **325**, 1127-1131 (1991).
- 3) Nomura, A., Stemmermann, G. N., Chyou, P. H., Kato, I., Perez-Perez, G. I. and Blaser, M. J. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. *N. Engl. J. Med.*, **325**, 1132-1136 (1991).
- 4) IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. *Helicobacter pylori*. In "Schistosomes, Liver Flukes and *Helicobacter pylori*: Views and Expert Opinions of an IARC Working Group on the Evaluation of Carcinogenic Risks to Humans," pp. 177-240 (1994). IARC, Lyon.
- 5) Webb, P. M., Yu, M. C., Forman, D., Henderson, B. E., Newell, D. J., Yuan, J.-M., Gao, Y.-T. and Ross, R. K. An apparent lack of association between *Helicobacter pylori* infection and risk of gastric cancer in China. *Int. J. Cancer*, **67**, 603-607 (1996).
- 6) Krakowka, S., Morgan, D. R., Kraft, W. G. and Leunk, R. D. Establishment of gastric *Campylobacter pylori* infection in the neonatal gnotobiotic piglet. *Infect. Immun.*, **55**, 2789-2796 (1987).
- 7) Radin, M. J., Eaton, K. A., Krakowka, S., Morgan, D. R., Lee, A., Otto, G. and Fox, J. *Helicobacter pylori* gastric infection in gnotobiotic beagle dogs. *Infect. Immun.*, **58**, 2606-2612 (1990).
- 8) Lee, A., Fox, J. G., Otto, G. and Murphy, J. A small animal model of human *Helicobacter pylori* active chronic gastritis. *Gastroenterology*, **99**, 1315-1323 (1990).
- 9) Karita, M., Kouchiyama, T., Okita, K. and Nakazawa, T. New small animal model for human gastric *Helicobacter pylori* infection: success in both nude and euthymic mice. *Am. J. Gastroenterol.*, **86**, 1596-1603 (1991).
- 10) Karita, M., Li, Q., Cantero, D. and Okita, K. Establishment of a small animal model for human *Helicobacter pylori* infection using germ-free mouse. *Am. J. Gastroenterol.*, **89**, 208-213 (1994).
- 11) Marchetti, M., Arico, B., Burroni, D., Figra, N., Rappuoli, R. and Ghiara, P. Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science*, **267**, 1655-1658 (1995).
- 12) Fox, J. G., Li, X., Cahill, R. J., Andrutis, K., Rustgi, A. K.,

- Odze, R. O. and Wang, T. C. Hypertrophic gastropathy in *Helicobacter felis*-infected wild type C57BL/6 mice and p53 hemizygous transgenic mice. *Gastroenterology*, **110**, 155–166 (1996).
- 13) Hirayama, F., Takagi, S., Yokoyama, Y., Iwao, E. and Ikeda, Y. Establishment of gastric *Helicobacter pylori* infection in Mongolian gerbils. *J. Gastroenterol.*, **31** (Suppl. IX), 24–28 (1996).
 - 14) Tatematsu, M., Katsuyama, T., Fukushima, S., Takahashi, M., Shirai, T., Ito, N. and Nasu, T. Mucin histochemistry by paradoxical concanavalin A staining in experimental gastric cancers induced in Wistar rats by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or 4-nitroquinone 1-oxide. *J. Natl. Cancer Inst.*, **64**, 835–843 (1980).
 - 15) Tatematsu, M., Ogawa, K., Hoshiya, T., Shichino, Y., Kato, T., Imaida, K. and Ito, N. Induction of adenocarcinomas in the glandular stomach of BALB/c mice treated with *N*-methyl-*N*-nitrosourea. *Jpn. J. Cancer Res.*, **83**, 915–918 (1992).
 - 16) Tatematsu, M., Yamamoto, M., Iwata, H., Fukami, H., Yuasa, H., Tezuka, N., Masui, T. and Nakanishi, H. Induction of glandular stomach cancers in C3H mice treated with *N*-methyl-*N*-nitrosourea in the drinking water. *Jpn. J. Cancer Res.*, **84**, 1258–1264 (1993).
 - 17) Yamamoto, M., Furihata, C., Fujimitsu, Y., Imai, T., Inada, K., Nakanishi, H. and Tatematsu, M. Dose-dependent induction of both pepsinogen-altered pyloric glands and adenocarcinomas in the glandular stomach of C3H mice treated with *N*-methyl-*N*-nitrosourea. *Jpn. J. Cancer Res.*, **88**, 238–244 (1997).
 - 18) Hirota, N., Aonuma, T., Yamada, S., Kawai, T., Saito, K. and Yokoyama, T. Selective induction of glandular stomach carcinoma in F344 rats by *N*-methyl-*N*-nitrosourea. *Jpn. J. Cancer Res. (Gann)*, **78**, 634–638 (1987).
 - 19) Sugimura, T. and Fujimura, S. Tumor production in glandular stomach of rat by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Nature*, **216**, 943–944 (1967).
 - 20) Sugimura, T., Fujimura, S. and Baba, T. Tumor production in the glandular stomach and alimentary tract of the rat by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Cancer Res.*, **30**, 455–465 (1970).
 - 21) Morson, B. C. Carcinoma arising from areas of intestinal metaplasia in the gastric mucosa. *Br. J. Cancer*, **9**, 377–385 (1955).
 - 22) Lauren, P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histoclinical classification. *Acta Pathol. Microbiol. Scand.*, **64**, 31–49 (1965).
 - 23) Ming, S. C., Goldman, H. and Freima, D. G. Intestinal metaplasia and histogenesis of carcinoma in human stomach. Light and electron microscopic study. *Cancer*, **20**, 1418–1429 (1967).
 - 24) Tatematsu, M., Ichinose, M., Miki, K., Hasegawa, R., Kato, T. and Ito, N. Gastric and intestinal phenotypic expression of human stomach cancers as revealed by pepsinogen immunohistochemistry and mucin histochemistry. *Acta Pathol. Jpn.*, **40**, 494–504 (1990).
 - 25) Tatematsu, M., Furihata, C., Katsuyama, T., Hasegawa, R., Nakanowatari, J., Saito, D., Takahashi, M., Matsushima, T. and Ito, N. Independent induction of intestinal metaplasia and gastric cancer in rats treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Cancer Res.*, **43**, 1335–1341 (1983).
 - 26) Tatematsu, M., Katsuyama, T., Furihata, C., Tsuda, H. and Ito, N. Stable intestinal phenotypic expression of gastric and small intestinal tumor cells induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or methylnitrosourea in rats. *Gann*, **75**, 957–965 (1984).
 - 27) Yuasa, H., Hirano, K., Kodama, H., Nakanishi, H., Imai, T., Tsuda, H., Imaida, K. and Tatematsu, M. Immunohistochemical demonstration of intestinal-type alkaline phosphatase in stomach tumors induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in rats. *Jpn. J. Cancer Res.*, **85**, 897–903 (1994).
 - 28) Parsonnet, J. The epidemiology of *C. pylori* infection. In “*Campylobacter pylori* in Gastritis and Peptic Ulcer Disease,” ed. M. J. Blaser, pp. 51–60 (1989). Igaku-Shoin, New York.