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Earlier *Helicobacter pylori* Infection Increases the Risk for the *N*-Methyl-*N*-nitrosourea-induced Stomach Carcinogenesis in Mongolian Gerbils

Xueyuan Cao,[1,2] Tetsuya Tsukamoto,[1,4] Koji Nozaki,[1,2] Harunari Tanaka,[1] Nobuyuki Shimizu,[2] Michio Kaminishi,[2] Toshiko Kumagai[3] and Masae Tatematsu[1]

[1]*Division of Oncological Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya, Aichi 464-8681,* [2]*Department of Gastrointestinal Surgery, Postgraduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033 and* [3]*Central Clinical Laboratories, Shinshu University, 3-1-1 Asahi, Matsumoto 390-8621*

***Helicobacter pylori* (*H. pylori*) is now well known to be associated with stomach cancer, with infection during childhood rather than as an adult considered to be more important for carcinogenesis. To evaluate the difference in susceptibility to stomach carcinogenesis in relation to age of acquisition of *H. pylori* infection, we designed an experiment involving inoculation of *H. pylori* ATCC43504 followed by *N*-methyl-*N*-nitrosourea (MNU) treatment at different ages. Four-week-old male Mongolian gerbils (MGs) were divided into twelve groups. *H. pylori* was inoculated at 4, 18 and 32 weeks of age, as representatives of early, middle and late infection, respectively. Two weeks later, the animals were treated with MNU. Groups without *H. pylori* and/or MNU were included as controls. The incidences of adenocarcinomas at 52 weeks after the inoculation in the early (*H. pylori*+MNU), middle (*H. pylori*+MNU), and late (*H. pylori*+MNU) group were 60% (12/20), 18.4% (2/11), and 10% (2/20), respectively. The corresponding figures were 14.8% (4/27), 0% (0/11), and 0% (0/21) in the MNU-alone groups. A higher titer of serum IgG for *H. pylori* and higher gastrin level were seen in the early-infected compared to the middle and the late groups ($P<0.01$). The results clearly demonstrated that early acquisition of *H. pylori* significantly increases gastric chemical carcinogenesis with MNU, as compared to the case with later infection, possibly because of differences in host gastric mucosal factors and immunologic responses.**

Key words: *Helicobacter pylori* — Gastric carcinogenesis — Mongolian gerbils

Helicobacter pylori (*H. pylori*) infection is the main cause of active chronic gastritis and peptic ulcer disease in both adults and children and the bacterium was classified as a group 1 carcinogen by WHO/IARC (World Health Organization/International Agency for Research on Cancer) in 1994.[1)] Recently, the study by Uemura *et al.* provided strong support for an intimate association between *H. pylori* infection and gastric disorders in man.[2)] Based on epidemiological data, age at acquisition of *H. pylori* infection appears to be an important factor influencing the degree of damage to gastric mucosa, and individuals infected with *H. pylori* when they are young have a higher relative risk for gastric cancer.[3, 4)] Our previous studies have proven that *N*-methyl-*N*-nitrosourea (MNU) and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) can induce glandular stomach cancers in Mongolian gerbils (MGs), and further demonstrated promoting effects due to *H. pylori* infection on tumor development.[5–7)] Since there has been no experimental investigation to determine the significance of *H. pylori* infection in children in relation to the risk of development of gastric cancer in adult life, we here examined the effects of *H. pylori* infection acquisition time on incidence of adenocarcinomas, serum anti-*H. pylori* IgG antibodies and gastrin levels in MGs.

[4] To whom requests for reprints should be addressed.
E-mail: ttsukamt@aichi-cc.jp

MATERIALS AND METHODS

Animals A total of 158 specific-pathogen-free male Mongolian gerbils (*Meriones unguiculatus*; MGS/Sea, Seac Yoshitomi, Ltd., Fukuoka), aged 4 weeks old, were housed in steel cages on hardwood chip bedding in an air-conditioned biohazard room with a 12 h light/12 h dark cycle. They were given food (Oriental MF; Oriental Yeast Co., Tokyo) and water *ad libitum*.

Bacterial inoculation *H. pylori* (ATCC 43504, American Tissue Culture Collection, Rockville, MD) was inoculated on *Brucella* agar plates (Becton Dickinson, Cockeysville, MD) containing 7% v/v 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 introduced into *Brucella* broth (Becton Dickinson) supplemented with 7% v/v heat-

inactivated fetal bovine serum and incubated under the same conditions for 24 h. Samples (0.8 ml) containing about 1.0×10^8 colony-forming units per milliliter were used as the inoculum for intragastric (i.g.) delivery via an oral catheter after the animals had been deprived of food for 24 h. Uninfected gerbils underwent sham inoculation using the same sterile *Brucella* broth.

Chemical MNU (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water at the concentration of 10 ppm (solutions were freshly prepared three times per week) for administration in light-shielded bottles as drinking water *ad libitum*.

Experimental protocol The experimental design, illustrated in Fig. 1, was approved by the Animal Care Committee of the Aichi Cancer Center Research Institute. The animals were treated as follows. One hundred and fifty-eight gerbils were divided into 12 groups. *H. pylori* was inoculated into six of these, two each at 0, 14, and 28 experimental weeks. The other 6 groups received *Brucella* broth. After 2 weeks, groups A, B, C, D, E, and F were given MNU in drinking water at the concentration of 10 ppm. Groups G, H, I, J, K, and L were given autoclaved distilled water continuously. The gerbils were sacrificed 52 weeks after *H. pylori* or *Brucella* broth inoculation. All animals were subjected to deep ether anesthesia after 24 h fasting, laparotomized, and exsanguinated from the inferior vena cava, followed by excision of their stomachs. Tissues fixed in 4% paraformaldehyde in phosphate-buffered saline were routinely processed for histopathological examination.

Histopathological analyses Tissue sections were stained with hemotoxylin and eosin (H&E) or with alcian blue (pH 2.5)-periodic acid Schiff (AB-PAS) for detection of mucin-containing cells, and with immunohistochemistry for *H. pylori* (anti-*H. pylori* serum, Dako, Copenhagen, Denmark). Adenocarcinomas of the glandular stomach were classified as "differentiated," characterized by tubular structures, and "undifferentiated" including signet-ring cell carcinomas.

Detection of *H. pylori* A piece of gastric mucosa (~30 mm^2) was taken from the pyloric area of each animal for detection of *H. pylori* infection. The samples were homogenized in *Brucella* broth and plated on segregating agar plates (Eiken Chemical Co., Tokyo) for culture of *H. pylori*, and incubated at 37°C under microaerobic conditions for 7 days.

Serology Serum samples were used to measure the titer of anti-*H. pylori* IgG antibodies (GAP-IgG; Biomerica, Newport Beach, CA) by enzyme-linked immunosorbent assay (ELISA) using anti-gerbil IgG antibodies. The antibody titer was expressed by means of an arbitrary index (AI). The cut-off value of anti-*H. pylori* IgG antibodies was used for judgement of the presence or absence of *H. pylori*. A value greater than 1.5 AI was considered to be positive for *H. pylori* infection in both the infection and the control groups as described.[8] Serum gastrin levels were measured using a gastrin radioimmunoassay kit (Gastrin-RIAkit II; Dainabot Co., Ltd., Tokyo).

Statistical analyses The incidences of adenocarcinomas were assessed by χ^2 and Fisher's exact probability methods. Anti-gerbil IgG antibody and gastrin levels are expressed as mean±SE values and comparisons between groups were made by using the Mann-Whitney *U* test. $P<0.05$ was considered to be statistically significant.

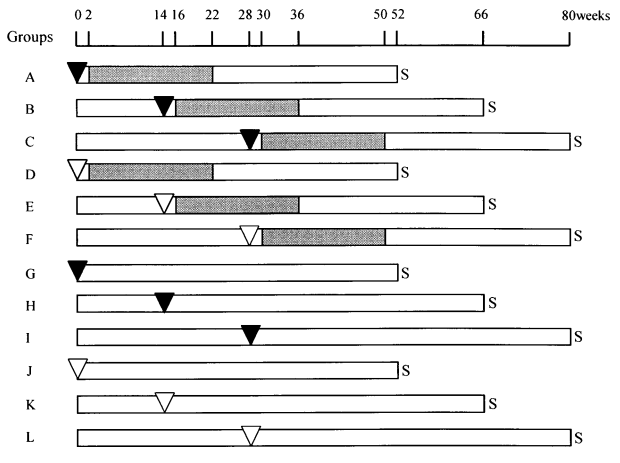

Fig. 1. Experimental design. Animals, 4-week-old male Mongolian gerbils; ▼, *H. pylori* (i.g.); ▽, *Brucella* broth (i.g.); ▭, MNU in drinking water at the concentration of 10 ppm; S, sacrifice.

RESULTS

Severity of infection At the end point of the experiment, survival rates for each group were >75%, with no differences among groups. Bacteriological examination showed no detectable *H. pylori* in uninfected MGs. Serum IgG titer was significantly higher in infected groups than in uninfected MGs. A higher titer of serum IgG for *H. pylori* was seen in group A compared to group C ($P<0.01$), and in group G as compared to group I ($P<0.01$) (Fig. 2A). The serum gastrin level was significantly greater in group A than in group B ($P<0.05$) or C ($P<0.01$); in the same way, it was significantly higher in group B than group C ($P<0.05$) (Fig. 2B). The early (Fig. 3A) *H. pylori*-infected gerbils had greater chronic active gastritis, lymphoplasmocytic infiltration and submucosal lymphoid follicle formation than the middle and late (Fig. 3B) *H. pylori*-infected groups.

Incidence of adenocarcinomas Histopathological findings are summarized in Table I. Whereas 12 of 20 (60%) in the early (*H. pylori*+MNU) group (group A) had adenocarcinomas in the glandular stomach, this was the case for

only 2 of 11 (18.2%) in the middle (*H. pylori*+MNU) group (group B), and 2 of 20 (10.0%) in the late (*H. pylori*+MNU) group (group C). The incidence of adenocarcinomas in group A was significantly higher than that in Group C (Table I, note *c*). Both well-differentiated (Fig. 4A) and signet ring-cell carcinomas (Fig. 4B) were found in group A gerbils. These neoplastic lesions were mainly located in the antrum or at the border between the antrum and the corpus. A trend for increase in the carcinoma incidence from group C, through B to A was also revealed to be statistically significant (Table I, note *e*).

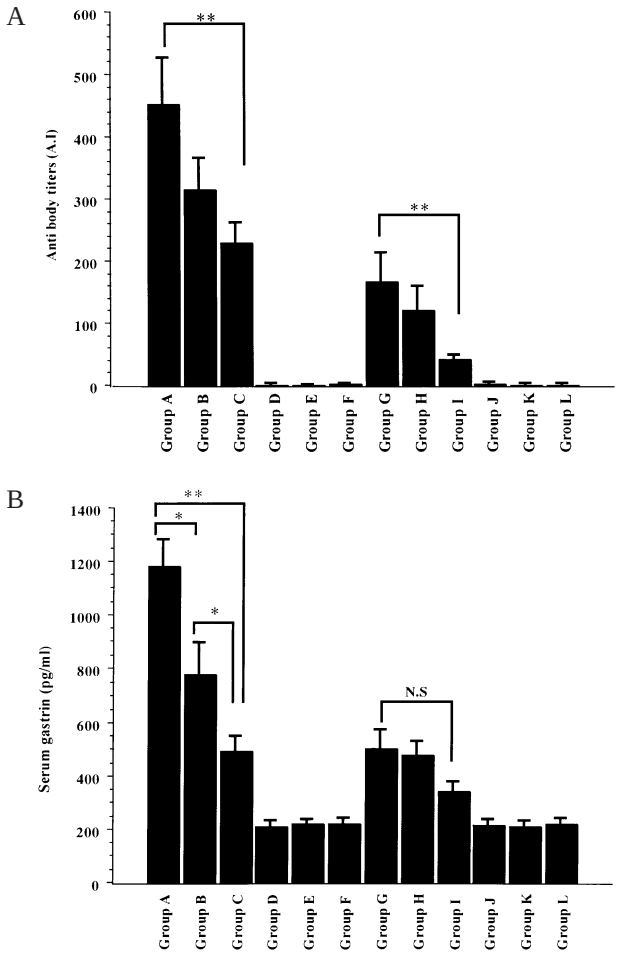

Fig. 2. (A) Changes in serum anti-*H. pylori* IgG titer in Mongolian gerbils (mean±SE). AI, arbitrary index. Serum anti-*H. pylori* IgG titer is significantly increased in group A as compared to group C, and in group G as compared to group I at 52 weeks after *H. pylori* inoculation. (B) Changes in serum gastrin level in Mongolian gerbils (mean±SE). Serum gastrin levels are significantly higher in group A than in groups B and C, and in group B than in group C. Error bar, ±1 standard error. ∗ $P<0.05$ and ∗∗ $P<0.01$. NS, no significant change, by Mann-Whitney *U* test.

In the early MNU-alone group (group D), 4 of 27 (14.8%) gerbils had adenocarcinomas in the glandular stomach, significantly lower than the incidence in group A ($P<0.01$, Table I, note *d*). In the middle and late MNU-alone groups (groups E and F), adenocarcinomas were not detected. In the *H. pylori* infection-alone groups (groups G, H and I), heterotopic proliferative glands (HPGs) were observed in all gerbils, but no tumors developed.

DISCUSSION

H. pylori infects about 50% of the human population, but only very few individuals develop gastric cancer. These observations suggest that some additional critical factors must interact with *H. pylori* to influence the course of stomach neoplasia. Age at acquisition of *H. pylori* infection is considered to be one important factor impacting on the degree of damage to gastric mucosa. In this study, we showed that early acquisition of *H. pylori* infec-

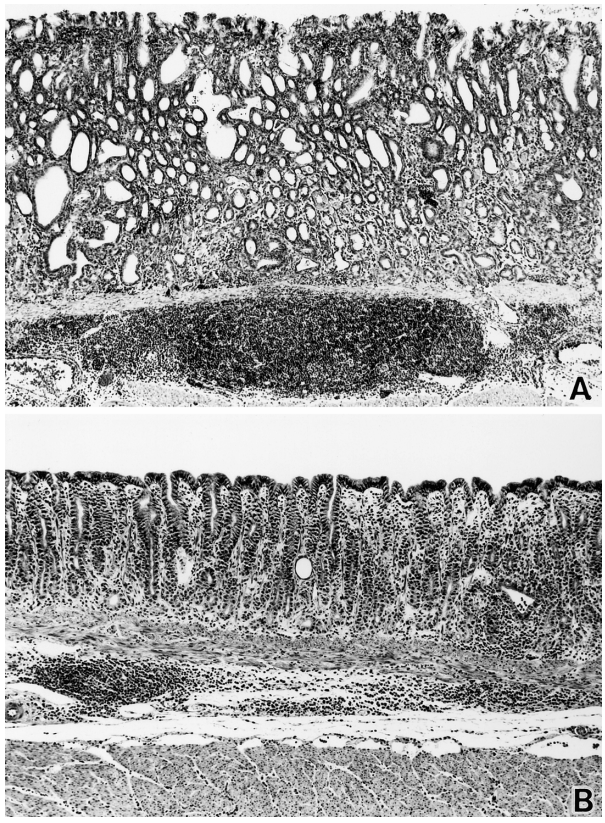

Fig. 3. Histopathological findings of pyloric mucosa 52 weeks after inoculation of *H. pylori*. (A) Marked chronic gastritis with lymphoid follicle formation in a group A gerbil (H&E, ×10). (B) Moderate gastritis with lymphoplasmocytic infiltration in a group C gerbil (H&E, ×10).

Table I. Incidence of Tumors in the Glandular Stomach of Mongolian Gerbils with *Hp* Infection and MNU Administration

Groups	Treatment[a)]	Effective no. of gerbils	Number of carcinoma-bearing gerbils (%)	Histology[b)]	
				Dif.	Undif.
A	Early (*Hp*→MNU)	20	12 (60.0)[c, d, e)]	10	2
B	Middle (*Hp*→MNU)	11	2 (18.2)	2	0
C	Late (*Hp*→MNU)	20	2 (10.0)	2	0
D	Early (*Br*→MNU)	27	4 (14.8)	4	0
E	Middle (*Br*→MNU)	11	0	0	0
F	Late (*Br*→MNU)	21	0	0	0
G	Early *Hp*	5	0	0	0
H	Middle *Hp*	5	0	0	0
I	Late *Hp*	5	0	0	0
J	Early *Br*	5	0	0	0
K	Middle *Br*	5	0	0	0
L	Late *Br*	4	0	0	0

a) *Hp*, *H. pylori* (i.g.); *Br*, *Brucella* broth (i.g.).
b) Dif., differentiated adenocarcinoma; Undif., undifferentiated adenocarcinoma.
c) $P<0.01$ versus group C.
d) $P<0.01$ versus group D.
e) $P<0.01$ among groups A, B and C.

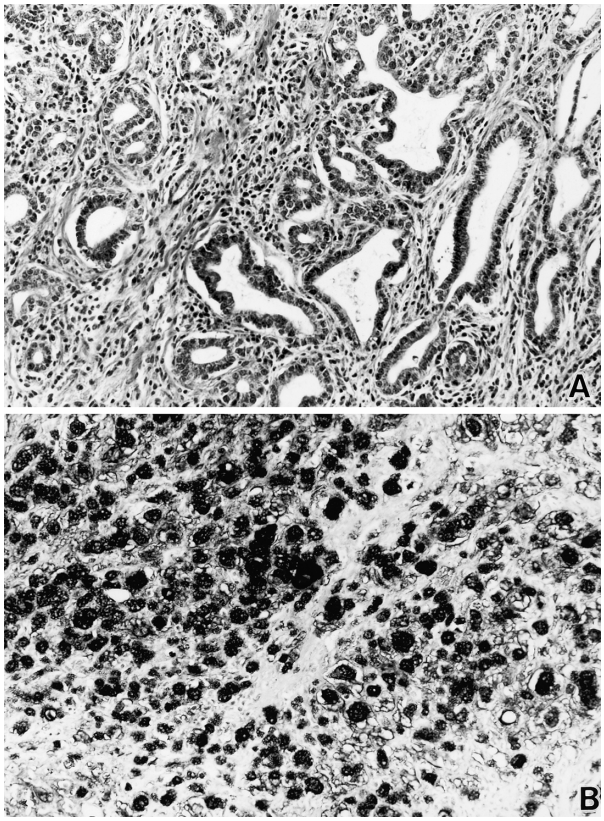

Fig. 4. (A) Well-differentiated adenocarcinoma in glandular stomach at week 52 in a group A gerbil (H&E, ×20). (B) Signet-ring carcinoma at week 52 in a group A gerbil (AB-PAS, ×40).

tion increases glandular stomach carcinoma risk. Thus, the incidence of adenocarcinomas was significantly higher in the early (*H. pylori*+MNU) group than with later infection and carcinogen exposure, or the early MNU-alone group. In addition, both well-differentiated adenocarcinoma and signet ring-cell carcinomas were found in the early (*H. pylori*+MNU) group, whereas only well-differentiated adenocarcinomas were detected in the middle and late (*H. pylori*+MNU) groups, implying differences in the level of carcinogenic insult.

The incidence of adenocarcinomas was significantly higher in the early (*H. pylori*+MNU) group A than the early MNU-alone group D. In the MNU-alone groups, adenocarcinomas were detected only in early exposed gerbils, indicating that MNU-induced chemical gastric carcinogenesis depends not only on the concentration,[5)] but also on the timing of administration in this model. Nonetheless, there was no significant difference in the incidences of gastric cancers among the MNU-alone groups D, E, and F. Thus, the early *H. pylori* infection was considered as a strong promoter for gastric carcinogenesis, even though administration of MNU at an early age also possibly contributed to the high incidence of adenocarcinomas in group A.

In the present study, we also found the titers of anti-*H. pylori* antibodies in early-infected animals to be higher than in the gerbils infected at a later age, presumably due to age-dependent variation in reaction to *H. pylori* antigens. Previous studies have shown that *H. pylori* infection induces cellular and humoral serum immune responses,

with T helper 1 cells contributing to *H. pylori*-associated gastritis in mice and humans.[9–11)]

The early *H. pylori*-infected gerbils in addition exhibited more severe lymphoplasmocytic infiltration and greater submucosal lymphoid follicle formation than in the middle and late *H. pylori*-infected groups. Previous studies indicated that children show unique antral nodularity, an important difference from *H. pylori*-associated disease in adults.[12)] Thus, there may be immunologic responses to local infection peculiar to animals and humans suffering early *H. pylori* infection.

Serum gastrin levels were also increased in gerbils of group A in the present study. Cytokines, such as interleukin 1β (IL-1β), play an important role in inflammatory and immunological responses,[13)] and recent studies have indicated that decrease in gastric acid secretion and increase in serum gastrin levels may be mediated by IL-1β in MGs infected with *H. pylori*.[14, 15)] In early *H. pylori*-infected gerbils, we consider that early infection may impede the development of parietal cells, causing compensational gastrin release, with a lower gastric acid secretion.[16)]

In the *H. pylori* infection-alone groups, both gastric-type and intestinal-type HPGs were observed. In our previous report we described the characteristics of the HPGs in the gastric mucosa of gerbils inoculated with long-term *H. pylori* alone.[17)] Eradication of *H. pylori* decreased HPG development. Although the HPGs showed features of hyperplasia and variable degrees of multifocal cystic dilatation, they can be readily distinguished from well-differentiated adenocarcinomas.[17)]

In conclusion, the present findings provide strong evidence that early acquisition of *H. pylori* infection increased the risk of glandular stomach cancer development, as compared with infection of gerbils at greater ages. This implied that early *H. pylori* infection could create a mucosal environment more susceptible to chemical carcinogenesis, with stronger promotion of neoplastic change. Further studies of genetic, epigenetic and microenvironmental alterations associated with differently timed infections are now warranted. Although many questions still remain regarding the efficacy of eradication, the current results indicate that childhood *H. pylori* infection must not be overlooked in approaches to the prevention of stomach cancer in adult life.[18, 19)]

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