Helicobacter pylori Does Not Promote *N*-Methyl-*N*-nitrosourea-induced Gastric Carcinogenesis in SPF C57BL/6 Mice

Yoshihiro Nakamura,[1] Takashi Sakagami,[1, 3] Noriyasu Yamamoto,[1] Yoshiro Yokota,[1] Hiromasa Koizuka,[1] Kazutoshi Hori,[1] Yoshihiro Fukuda,[1] Noritoshi Tanida,[1] Takehiko Kobayashi[2] and Takashi Shimoyama[1]

[1]*Department of Gastroenterology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501 and* [2]*Department of Chemotherapy, Tokyo Metropolitan Komagome Hospital, 3-18-22 Honko-magome, Bunkyo-ku, Tokyo 113-0012*

***Helicobacter pylori* (*H. pylori*) infection has been acknowledged as a promoter and an initiator for gastric carcinogenesis in experimental models using Mongolian gerbils with *H. pylori* strains TN2GF4 and ATCC 43504, which have +ve *cag*A and *vac*A phenotype s1/m1. To get more insight into the role of *H. pylori* in gastric carcinogenesis, we studied the effect of *H. pylori* SS1, which has +ve *cag*A and *vac*A phenotype s2/m2, on *N*-methyl-*N*-nitrosourea (MNU)-induced chemical gastric carcinogenesis using SPF C57BL/6 mice. Thus, *H. pylori* SS1 was inoculated 1 week after the completion of MNU treatment to examine the promoting effect of this bacterium. The incidences of polypoid lesions, differentiated adenocarcinomas, and adenomatous hyperplasias were 67% (10/15), 47% (7/15) and 80% (12/15), respectively, in the MNU-alone group. The corresponding figures were 31% (8/26), 23% (6/26) and 35% (9/26) in the MNU+*H. pylori* group. The incidences of polypoid lesions and adenomatous hyperplasia were significantly different between the groups. Thus, the results indicate that *H. pylori* SS1 infection reduced susceptibility to chemical gastric carcinogenesis in this model. The discrepancy between the present result and previous results is likely to have been caused by differences in host factors and bacterial factors. Further study of the relationship between gastric carcinogenesis and *H. pylori* infection is needed.**

Key words: *Helicobacter pylori* — Gastric carcinogenesis — Mouse model — MNU

The International Agency for Research on Cancer (IARC), a working group of the WHO, acknowledged *Helicobacter pyroli* (*H. pylori*) as a group 1 definite carcinogen of gastric cancer in 1994.[1] What was emphasized at that juncture was that the decision was based on epidemiological data.[2–4] Virtually no other evidence was available about gastric carcinogenesis in relation to *H. pylori* at the time. Furthermore, the epidemiological data did not show a consistent relation between gastric cancer and *H. pylori* infection, as, for example, in the African enigma, wherein gastric cancer rates were low despite a very high prevalence of *H. pylori* infection in African countries.[5] This discrepancy suggests that some host factors and/or bacterial factors might modify the gastric carcinogenesis associated with *H. pylori* infection. Therefore further study was necessary to evaluate the relation between *H. pylori* infection and gastric carcinogenesis. We conducted several rodent experiments to study the carcinogenic role of *Helicobacter*, but all failed to show any carcinogenic effect.[6, 7] In 1998, *H. pylori* infection without any other treatment was reported to induce gastric carcinogenesis in Mongolian gerbils by Watanabe *et al.* from Japan.[8] This result

suggested that *H. pylori* infection does play a major role in gastric carcinogenesis. However, it should be pointed out that "gastric cancer" induced by *H. pylori* resolved spontaneously when *H. pylori* was eradicated from these animals.[9] The latter study brought into question the role of *H. pylori* as an initiator in gastric carcinogenesis. Nonetheless, Sugiyama *et al.* provided evidence that *H. pylori* had a promoting effect on chemical gastric carcinogenesis in Mongolian gerbils.[10] This information may suffice to link *H. pylori* infection and gastric carcinogenesis. However, it remains necessary to study the role of *H. pylori* in gastric carcinogenesis using other hosts under well-defined conditions and other types of *H. pylori* strain. For this purpose, we employed the mouse model developed by Tatematsu *et al.* for experimental gastric carcinogenesis.[11] Our previous studies using that mouse model did not show an enhancing effect of *H. pylori* (strain SS1) in gastric carcinogenesis.[7] Rather, a suppressive effect of *H. pylori* was indicated in our experiments. The incidence of gastric neoplasma in our previous models was 5.6% to 30% in animals given *N*-methyl-*N*-nitrosourea (MNU), which might not be appropriate to see any promoting effect of a co-carcinogen. Therefore, in the present study, we used the experimental procedure for inducing gastric carcinogenesis with MNU reported by Yamachika *et al.*, which afforded a high inci-

---

3 To whom correspondence should be addressed.
  E-mail: sakan@hyo-med.ac.jp

dence of gastric carcinoma in mice.[12] Thus, we studied whether or not *H. pylori* (strain SS1) infection enhanced susceptibility to MNU-induced gastric carcinogenesis in the SPF C57BL/6 mouse model.

MATERIALS AND METHODS

Animal A total of fifty 6-week-old male SPF C57BL/6 mice (Charles River, Inc., Atsugi) were housed in plastic cages on wood chips in an air-conditioned biohazard room with a 12 h light-12 h dark cycle. They were fed on a diet of sterilized commercial pellets (CE-2, Clea Japan, Inc., Tokyo) and autoclaved distilled water was given *ad libitum*.
Chemical MNU (Sigma Chemical Co., St Louis, MO) was dissolved in distilled water at the concentration of 200 p.p.m. (freshly prepared 2 times per week), and provided as the drinking water *ad libitum* on alternate weeks for 10 weeks (total exposure: 5 weeks) in light-shielded bottles. All mice were given MNU treatment.
Bacterium *H. pylori*, strain SS1, *cag*A +ve, *vac*A phenotype s2/m2 human isolate adapted to colonize the mouse stomach, was grown in brucella broth containing lysed horse blood 10% v/v and supplemented with vancomycin 10 mg/liter and polymyxin B 2500 i.u./liter. These broths were incubated for 20 h under a microaerophilic condition (15% CO_2) at 37°C. *H. pylori* were adjusted to the concentration of 1.5×10^9 CFUs/0.5 ml, and 0.5 ml of suspension was inoculated orally using a stomach tube 3 times a

week. Uninfected animals had sham inoculation using the same sterile brucella broth.
Experimental schedule Fig. 1 shows the experimental schedule. All mice were divided into two groups after the MNU treatment. Thirty mice were inoculated with *H. pylori* SS1 1 week after the completion of the MNU treatment (MNU+*H. pylori* group). Twenty of them were without *H. pylori* SS1 inoculation (MNU-alone group). All animals were sacrificed under anesthesia with diethyl ether at week 54 and histopathological changes of the gastric mucosa and colonization of *H. pylori* were assessed. All protocols in this study were conducted in accordance with the institutional animal experimentation guidelines.
Histopathological assessment The stomach was removed and the greater curvature was opened. The stomach was extended on a wooden plate and the mucosa was carefully examined for protruding lesions. Any protruding lesion was reserved for further histological examination. Thereafter a small part of the stomach was used for culture of *H. pylori*. The stomach specimens were fixed in 10% neutral buffered formalin and were cut along the longitudinal axis into 8 slices. These slices and those including protruding lesions were embedded in paraffin wax, and 5 μm thick sections were cut. These sections were stained with hematoxylin and eosin and periodic acid-Schiff reagent for histopathological assessment.

Macroscopically, polypoid lesions larger than 2 mm × 2 mm × 2 mm (longitudinal axis × minor axis × height) were considered as tumors. All specimens were examined by our pathologist (K. H.), who was unaware of the experimental protocol.

Neoplastic findings were classified as follows: (i) tubular adenocarcinoma, dysplastic tubules crowded with disorganized architecture, composed primarily of acinar structures with cribriform and/or cystic appearance. The cells had enlarged vesicular nuclei with prominent nucleoli, scant cytoplasms, and showed loss of polarity, mucin depletion and a number of mitoses. The nuclei were hyperchromatic, plemorphic or stratified; (ii) adenomatous hyperplasia, elongated tubules with branching, distorted or serrated configurations. The cells had no atypia and abundant cytoplasms; (iii) hyperplastic nodules, enlonged
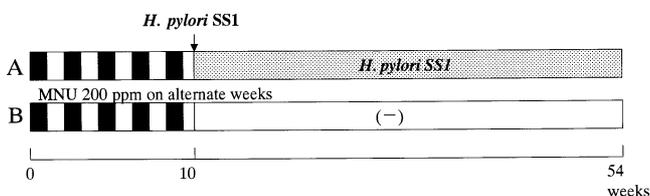

Fig. 1. Experimental protocol. A: MNU at 200 ppm for alternate weeks given for 10 weeks (total exposure: 5 weeks). Animals were inoculated with *H. pylori* SS1 1 week after the completion of MNU treatment. B: MNU treatment for 10 weeks without *H. pylori* SS1 inoculation.

Table I. Histopathological Findings. Incidence of Tumorigenesis in Mice Treated with MNU with and without *H. pylori* SS1 Infection

Experimental group	Histopathology			
	Polypoid lesion	Adenocarcinoma[a]	Adenomatous hyperplasia	Severe gastritis
MNU+*H. pylori* (n=26)	8/26 (31)[b]	6/26 (23)	9/26 (35)	5/29 (19)
MNU-alone (n=15)	10/15 (67)*	7/15 (47)	12/15 (80)*	0/15 (0)

a) Well-differentiated adenocarcinoma.
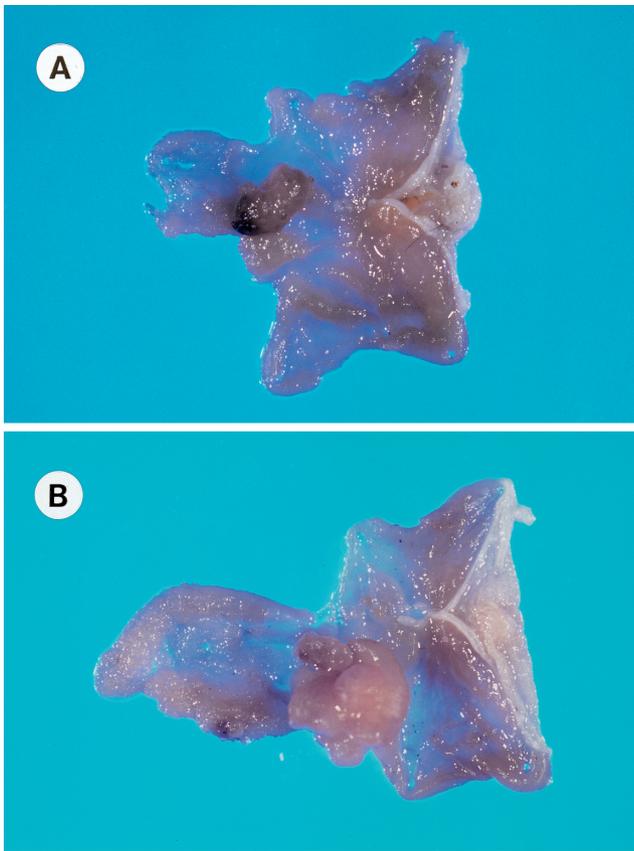b) ( ) is percentage.
* $P<0.05$.

Fig. 2. Macroscopic findings in stomach of C57BL/6 mice. A: MNU+*H. pylori* SS1 group. Polypoid lesion in the antrum. B: MNU-alone group. Polypoid lesion in the antrum.
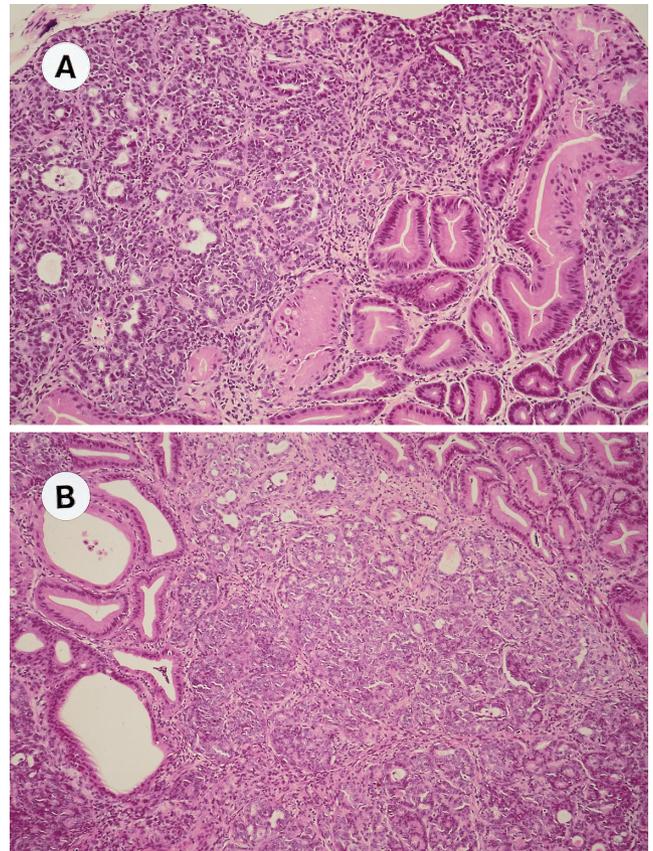

Fig. 3. Microscopic findings in glandular stomach of C57BL/6 mice. A: MNU+*H. pylori* SS1 group. Well-differentiated adenocarcinoma in adenomatous hyperplasia. The carcinoma cells are present in smaller tubules with focally acinar structure and cribriform appearance in contrast to the surrounding tubules with serrated configuration, and have hyperchromatic nuclei and scant cytoplasm. B: MNU-alone group. Well-differentiated adenocarcinoma in adenomatous hyperplasia. Dysplastic tubules with acinar structure, of which some show an alveolar pattern and infiltrate into hyperplastic tubules with cystic or serrated configuration. A and B are the same as those in Fig. 2.

tubules without cellular and architectonic atypia. Gastritis was categorized into (i) severe gastritis, thickened mucosa with severe infiltration of inflammatory cells; (ii) moderate gastritis, thickened mucosa with moderate infiltration of inflammatory cells; (iii) mild gastritis, mucous, not thickened mucosa with moderate infiltration of inflammatory cells.

Detection of *H. pylori* Infestation of *H. pylori* was assessed bacteriologically and serologically. A part of the stomach mucosa was incubated in *Campylobacter* Selective Agar in our laboratory. Systemic IgG antibody against *H. pylori* in the serum was measured using an enzyme-linked immunosorbent assay adapted from that of Evans *et al.*[13] The antigen of *H. pylori* was high-molecular-weight, cell-associated protein of *H. pylori* 43504 (Kyowa Medix, Tokyo). Sample sera were diluted 100 times with blocking buffer and specific antibody against *H. pylori* was detected by using peroxidase-conjugated anti-mouse IgG (Dako Japan, Tokyo) as the second antibody. The absorbance was measured at 450 nm.

Statistics The incidences of neoplastic lesions were assessed by Fisher's exact probability test. The two-tailed *t* test was used to assess the significance of differences in the absorbance value to evaluate *H. pylori*-specific IgG in the serum. The criterion for statistical significance was $P<0.05$.

RESULTS

Thirteen percent and 25% of mice had died by the end of the experiment in the MNU+*H. pylori* group and MNU-alone group, respectively (statistically insignificant). The colonization of *H. pylori* was detected bacteriologi-

cally in all mice, which were given *H. pylori*-containing broth. A higher titer of serum IgG for *H. pylori* was seen in them compared to uninfected animals (absorbance value, 0.12±0.082 vs. 0.04±0.01, *P*<0.01).

Histopathological findings are summarized in Table I. Macroscopically, 8 of 25 (31%) in the MNU+*H. pylori* group had polypoid lesions in the stomach, compared to 10 of 15 (67%) in the MNU-alone group. These polypoid lesions were distributed at the antrum and/or the border of the antrum and corpus (Fig. 2). The diameter of these tumors ranged from 3 to 5 mm. Some of them had ulceration. There was a significant difference in the incidences of polypoid lesions between the MNU+*H. pylori* group and MNU-alone group (*P*<0.05). Microscopically, 6 in the MNU+*H. pylori* group (23%) and 7 in the MNU-alone group (47%) had differentiated adenocarcinoma (Fig. 3). Nine in the MNU+*H. pylori* group (35%) and 12 in the MNU-alone group (80%) had adenomatous hyperplasia (*P*<0.05). All carcinomas, which were diagnosed on the basis of cellular and architectonic atypical appearance, existed next to adenomatous hyperplasia. These carcinomas did not penetrate the muscular layer.

With regard to the severity of gastritis, 9 in the MNU+*H. pylori* group (35%) and 10 in the MNU group (67%) had moderate gastritis. In the MNU+*H. pylori* group, 5 (19%) had severe infiltration of inflammatory cells characterized by dense neutrophil and mononuclear cells. These findings were noted near adenomatous hyperplasia and/or differentiated adenocarcinoma. Severe gastritis was not observed in the MNU-alone group. Complete intestinal metaplasia was not observed, but incomplete intestinal metaplasia was detected in both groups. The severity of these lesions was mild and the density was low.

DISCUSSION

In 1998, gastric carcinogenesis in an animal model due to *H. pylori* infection alone was first reported by Watanabe *et al.* using Mongolian gerbils (MGS/Sea).[8] Honda *et al.* also reported the development of gastric differentiated adenocarcinoma in *H. pylori*-infected Mongolian gerbils (MGS/Sea).[14] The nature of the "adenocarcinoma" developed in *H. pylori*-infected Mongolian gerbils has been questioned because spontaneous regression occurred after eradication of *H. pylori*.[9] Furthermore, it should be emphasized that all findings on *H. pylori*-induced "adenocarcinoma" were obtained in a single strain of Mongolian gerbils (MGS/Sea). This animal has naturally occurring *Helicobacter hepaticus* in its gastrointestinal tract.[15] It has been reported that the development of dysplasia and carcinoma of stomach are influenced by natural *Helicobacter* infection (*Helicobacter muridarum*) in a conventional Quankenbush Swiss strain mouse model.[16] These findings suggest that we need to study the effect of host

factors and bacterial factors on *H. pylori*-induced gastric carcinogenesis in detail.

We have already conducted several experiments in mice to investigate the role of *H. pylori* infection in gastric carcinogenesis. We tested whether atrophic gastritis due to *Helicobacter* infection resulted in a higher susceptibility to chemical gastric carcinogenesis (MNU) using *Helicobacter felis*-infected conventional QS strain mice.[6] Gastric mucosal cell proliferation was accelerated in infected animals, but the incidence of gastric cancer did not differ significantly between infected and uninfected animals. We have also investigated the promoting effect of *H. pylori* infection using SPF Balb/C and SPF ICR mice, *H. pylori* SS1, and MNU as a chemical carcinogen.[7] Again the incidence of adenomatous hyperplasia was similar in the infected and uninfected groups. Danon and Eaton investigated whether gastric epithelial proliferation due to *Helicobacter* infection enhanced susceptibility to chemical gastric carcinogenesis using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine in *Helicobacter heilmannii*-infected mice.[17] They concluded that the gastric proliferative lesion in *H. heilmannii*-infected mice was not pre-neoplastic, so *Helicobacter* infection was not a condition for higher susceptibility to gastric carcinogenesis in this model. These findings suggested that *Helicobacter* was not an initiator or a promoter for gastric carcinogenesis in these mouse models, when *H. felis* and *H. heilmannii* were used as bacteria and Balb/C and ICR mice were used as experimental animals.

In the present study, SPF C57BL/6 mice and *H. pylori* SS1 were used. *H. pylori* SS1 infected various strains of mice and induced gastric mucosal changes.[18, 19] In our experience, C57BL/6 mice had good colonization during the entire observation period of at least 18 months. Mild infiltration of neutrophils and lymphocytes were observed in the lamina propria at 3 months after inoculation. Acute gastritis progressed to chronic active gastritis. Thus, lymphocyte infiltration was observed with time; the finding was especially conspicuous in the deep portion of the mucosa and the submucosal layer. This continued during the following experimental period of 18 months. Intestinal metaplasia, peptic ulcer and adenocarcinoma were not detected at the end of this experiment. The severity of gastric mucosal changes was remarkable in SPF C57BL/6 mice compared to mice of other strains. Therefore, C57BL/6 mice and *H. pylori* SS1 strain were most appropriate to study additional effects of *H. pylori* in chemical carcinogenesis.

The MNU-induced mouse gastric carcinogenesis model, which was developed by Tatematsu *et al.*, has been used successfully and extensively to study carcinogenesis of the stomach. Our results were consistent with those of previous studies and carcinogenesis was induced successfully in C57BL/6 mice. A total of 13 carcinomas developed in the

present experiment. There was no significant difference in the incidence of adenocarcinomas between the MNU+*H. pylori* group and MNU-alone group. However the incidences of polypoid lesion and adenomatous hyperplasia were higher in the MNU-alone group. Thus, *H. pylori* infection did not enhance susceptibility to the chemical gastric carcinogen (MNU) in the SPF C57/BL6 mouse model, but rather tended to inhibit gastric carcinogenesis. At variance with our result, Sugiyama *et al.* reported that *H. pylori* infection enhanced susceptibility to chemical gastric carcinogenesis in Mongolian gerbils (MGS/Sea).[10] The reason for the discrepancy between the present study and the Mongolian gerbil model seems critical in evaluating the role of *H. pylori* in gastric carcinogenesis. One possible reason is the difference in the severity of gastric mucosal changes between those two models.

Mongolian gerbils infected with *H. pylori* had very severe inflammatory cell infiltration, ulceration and intestinal metaplasia in the mucosal layer.[20] In contrast, moderate-mild chronic active gastritis and no ulceration were observed in SPF C57/BL6 mice infected with *H. pylori*.[18, 19] Shimizu *et al.* reported that *H. pylori* infection promoted development of pepsinogen-altered pyloric glands, a preneoplastic lesion of the glandular stomach, in Balb/C mice treated with MNU.[21] They used *H. pylori* strain C0014 as the bacterium. This bacterium has +ve *cag*A and VacA activity (personal communication from K. Imagawa, Otsuka Pharmaceutical). In addition, strains

TN2GF4 and ATCC 43504, which have +ve *cag*A and *vac*A phenotype s1/m1 were used as bacteria in the Mongolian gerbils experiments. In the present study, we used *H. pylori* SS1, which has +ve *cag*A and *vac*A phenotype s2/m2,[22] because this was the only strain which showed persistent long-term colonization in mice.[18, 19] The *vac*A phenotype s2/m2 does not have vacuolating activity.[23] Some epidemiological reports suggested that vacA protein (vacuolating activity) was a virulence factor of *H. pylori*.[24, 25] Thus, the difference in *H. pylori* strains might have affected gastric carcinogenesis in these animal models. If this were the case, our result seems to suggest that non-pathogenic *H. pylori* strains have the ability to suppress carcinogenesis. Future study on bacterial factors and host factors may give more insight into the role of *H. pylori* in gastric carcinogenesis.

REFERENCES

1) International Agency for Research on Cancer, World Health Organization. Schisosomes, liver flukes and *Helicobacter pylori*. *IARC Monogr. Eval. Carcinog. Risks Hum.*, **61**, 177–241 (1994).

2) Forman, D., Newell, D. G., Fullerton, F., Yarnell, J. W., Stacey, A. R., Wald, N. and Sitas, F. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. *BMJ*, **1**, 1302–1305 (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) 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).

5) Holcombe, C. *Helicobacter pylori*: the African enigma. *Gut*, **33**, 429–431 (1992).

6) Sakagami, T., Sawada, Y., Hikasa, H., Tanida, N., Shimoyama, T. and Lee, A. Exposure of long-term *Helicobacter felis* infected mice to the carcinogen N-methyl-N-nitrosourea. *Recent Advances in Gastroenterological Carcinogenesis I*, 461–464 (1996).

7) Sakagami, T., Akashi, H., Sawada, Y., Fukuda, Y., Tanida, N., Shimoyama, T., Nishigami, T., O'Rourke, J. and Lee, A. Effect of *Helicobacter* infection in chemically induced gastric carcinogenesis in mice. *Gut*, **41** (Suppl. 1), A117 (1997).

8) Watanabe, T., Tada, M., Nagai, H., Sasaki, S. and Nakao, M. *Helicobacter pylori* infection induces gastric cancer in mongolian gerbils. *Gastroenterology*, **115**, 642–648 (1998).

9) Nozaki, K., Shimizu, N. and Tatematsu, M. Eradication diminishes submucosal proliferating lesions induced by *Helicobacter pylori* infection in glandular stomach of Mongolian gerbils. *Gastroenterology*, **32**, 44–46 (2001) (in Japanese).

10) Sugiyama, A., Maruta, F., Ikeno, T., Ishida, K., Kawasaki, S., Katsuyama, T., Shimizu, N. and Tatematsu, M. *Helicobacter pylori* infection enhances N-methyl-N-nitrosourea-induced stomach carcinogenesis in the Mongolian gerbil. *Cancer Res.*, **58**, 2067–2069 (1998).

11) 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).

12) Yamachika, T., Nakanishi, H., Inada, K., Tsukamoto, T., Shimizu, N., Kobayashi, K., Fukushima, S. and Tatematsu, M.   *N*-Methyl-*N*-nitrosourea concentration-dependent, rather than total intake-dependent, induction of adenocarcinomas in the glandular stomach of BALB/c mice. *Jpn. J. Cancer Res.*, **89**, 385–391 (1998).

13) Evans, D. J., Jr., Evans, D. G., Graham, D. Y. and Klein, P. D. A sensitive and specific serologic test for detection of *Campylobacter pylori* infection. *Gastroenterology*, **96**, 1004–1008 (1989).

14) Honda, S., Fujioka, T., Tokieda, M., Satoh, R., Nishizono, A. and Nasu, M. Development of *Helicobacter pylori*-induced gastric carcinoma in Mongolian gerbils. *Cancer Res.*, **58**, 4255–4259 (1998).

15) Sakagami, T., Yamamoto, N., Sashio, H., Sawada, Y., Tamura, K., Fukuda, Y., Tanida, N. and Shimoyama, T. *Helicobacter pylori* and carcinogenesis experiments. *In* "*Helicobacter pylori*. Infection in Gastroduodenal Lesions, The Second Decade," ed. J. M. Pajares Garcia, P. Correa and G. I. Perez, pp. 295–303 (2000). Prous Science, Spain.

16) Lee, A., Correa, P., Roa, J., Leung, V., O'Rourke, J. and Dixon, M. F. The first evidence of dysplasia and possibly gastric cancer caused by natural *Helicobacter* infection in an animal model. *Gut*, **43** (Suppl. 2), A66–A67 (1998).

17) Danon, S. J. and Eaton, K. A. The role of gastric *Helicobacter* and N-methyl-N′-nitro-N-nitrosoguanidine in carcinogenesis of mice. *Helicobacter*, **3**, 260–288 (1998).

18) Sakagami, T., Dixon, M., O'Rourke, J., Howlett, R., Alderuccio, F., Vella, J., Shimoyama, T. and Lee, A. Atrophic gastric changes in both *Helicobacter felis* and *Helicobacter pylori* infected mice are host dependent and separate from antral gastritis. *Gut*, **39**, 639–648 (1996).

19) Lee, A., O'Rourke, J., De Ungria, M. C., Robertson, B., Daskalopoulos, G. and Dixon, M. F. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology*, **112**, 1386–1397 (1997).

20) Sawada, Y., Kuroda, Y., Sashio, H., Yamamoto, N., Tonokatsu, Y., Sakagami, T., Fukuda, Y., Shimoyama, T., Nishigami, T. and Uematsu, K. Pathological changes in glandular stomach of *Helicobacter pylori*-infected Mongolian gerbil model. *J. Gastroenterol.*, **33** (Suppl. 10), 22–25 (1998).

21) Shimizu, N., Kaminishi, M., Tatematsu, M., Tsuji, E., Yoshikawa, A., Yamaguchi, H., Aoki, F. and Oohara, T. *Helicobacter pylori* promotes development of pepsinogen-altered pyloric glands, a preneoplastic lesion of glandular stomach of BALB/c mice pretreated with N-methyl-N-nitrosourea. *Cancer Lett.*, **123**, 63–69 (1998).

22) Salaun, L., Audibert, C., Le Lay, G., Burucoa, C., Fauchere, J. L. and Picard, B. Panmictic structure of *Helicobacter pylori* demonstrated by the comparative study of six genetic markers. *FEMS Microbiol. Lett.*, **161**, 231–239 (1998).

23) Atherton, J. C., Cao, P., Peek, R. M., Jr., Tummuru, M. K., Blaser, M. J. and Cover, T. L. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific vacA types with cytotoxin production and peptic ulceration. *J. Biol. Chem.*, **270**, 17771–17777 (1995).

24) Figura, N., Guglielmetti, P., Rossolini, A., Barberi, A., Cusi, G., Musmanno, R. A., Russi, M. and Quaranta, S. Cytotoxin production by *Campylobacter pylori* strains isolated from patients with peptic ulcers and from patients with chronic gastritis only. *J. Clin. Microbiol.*, **27**, 225–226 (1989).

25) Zhang, Q. B., Nakashabendi, I. M., Mokhashi, M. S., Dawodu, J. B., Gemmell, C. G. and Russell, R. I. Association of cytotoxin production and neutrophil activation by strains of *Helicobacter pylori* isolated from patients with peptic ulceration and chronic gastritis. *Gut*, **38**, 841–845 (1996).