J. Pharm. Pharmacol. 1998, 50: 943–948
Received September 5, 1997
Accepted March 23, 1998

© 1998 J. Pharm. Pharmacol.

Delayed Healing of Chronic Gastric Ulcer after *Helicobacter pylori* Infection in Mice

MITSUKO MAKINO, TETSUFUMI KOGA*, KEIICHI ITO, HARUMI KAWADA* AND KEIICHI TABATA

*Pharmacology and Molecular Biology Research Laboratories and *Biological Research Laboratories, Sankyo Co. Ltd, Tokyo, Japan*

Abstract

It has been suggested that there is a close relationship between *Helicobacter pylori* and the onset or recurrence of gastroduodenal disease. The aim of this study was to examine the effect of *H. pylori* on the healing of chronic gastric ulcers induced in mice.

H. pylori administered to nude mice delayed the healing of experimental acetic acid-induced gastric ulcers. Histological examination showed the occurrence of high densities of *H. pylori* on the surface of epithelial cells and in the ulcerated area. Repeated administration of amoxicillin (10 mg kg^{-1} daily for 5 days) eradicated *H. pylori* and increased the rate of healing of gastric ulcers in *H. pylori*-infected mice, but metronidazole, which also eradicated the organisms, did not significantly affect the rate of healing.

In conclusion, *H. pylori*-infection delayed the healing of gastric ulcers induced by the serosal application of acetic acid in mice, possibly by aggravation or prolongation of the mucosal inflammation. Amoxicillin eradicated *H. pylori* and promoted gastric ulcer healing in mice infected with *H. pylori*.

Helicobacter pylori, a Gram-negative spiral bacterium, was first isolated from a patient with chronic gastritis by Warren & Marshall in 1983. Since then, much evidence has indicated the close relationship between gastroduodenal disease and *H. pylori* (Graham et al 1987; Marshall & Langton 1986). Eradication of *H. pylori* by combined treatment with antibiotics and antacids is followed by a reduced rate of recurrence of peptic ulcers and slow resolution of the underlying gastritis (Marshall et al 1988; Perterson 1991; Graham et al 1992). It is therefore assumed that *H. pylori* might be an important pathogenic factor in ulcer recurrence. However, to clarify the clinical significance of *H. pylori* in ulcer healing the healing of gastric ulcer should be deleteriously influenced by continuous infection.

Several species such as mice (Karita et al 1994), pigs (Krakowka et al 1987; Engstrand et al 1990) and monkeys (Baskerville & Newell 1988; Shuto et al 1993; Fujioka et al 1994) have recently been used as *H. pylori* infection models. Karita et al (1993, 1994) established chronic infection of *H. pylori* in athymic mice in which *H. pylori* continuously colonized in the stomach. Other studies (Ross et al 1992; Karita et al 1994) have focused on the development of experimental acute and chronic gastritis with *H. pylori*. Despite this work it remains unclear whether *H. pylori* hinders ulcer healing in experimental animals.

This study was performed to study the healing of gastric ulcers induced in nude mice, and the effect of antibacterial drugs in *H. pylori*-infected animals.

Correspondence: K. Tabata, Pharmacology and Molecular Biology Research Laboratories, Sankyo Co. Ltd, 1-2-58 Hiromachi, Shinagawa, Tokyo 140, Japan.

Materials and Methods

Chemicals

Acetic acid was from Iwai Kagaku (Tokyo, Japan), amoxicillin from Kyowa Hakko Kogyo (Tokyo, Japan), metronidazole, vancomycin and nalidixic acid from Sigma (St Louis, MO), polymyxin B from Pfizer Pharmaceutical (Tokyo, Japan), trimethoprim from Shionogi Pharmaceutical (Osaka, Japan) and 3 mg mL^{-1} amphotericin B from Bristol-Myers Squibb (Tokyo, Japan).

Animals

BALB/C athymic nude mice, 6 weeks, were obtained from Nippon SLC (Shizuoka, Japan); for

at least 1 week before use they were maintained on chow sterilized by radiation (Funabashi, Chiba, Japan). Sterile water was freely available.

Induction of gastric ulcer

Gastric ulcers were induced by serosal application of acetic acid, according to the method of Takagi et al (1969). Mice were anaesthetized with sodium pentobarbital (30 mg kg^{-1}, i.p.). The abdomen was opened and the stomach exteriorized. A metal cup (3 mm diam.) was placed at the anterior border between the corpus and antrum, and acetic acid solution (60%; 0·2 mL) was poured into the cup to make contact with the surface of the stomach. After 15 s the cup was removed, the acetic acid solution was removed by absorption into filter paper, the surface of the stomach was washed with sterile saline and the abdomen was closed by suture. Thereafter, the animals were maintained as described above.

Bacteria

A clinical isolate (CPY 2052) was kindly supplied by Dr Mikio Karita (School of Medicine, Yamaguchi University, Yamaguchi, Japan). The strain was identified by morphology, Gram's stain, urease, oxidase, catalase production, resistance to nalidixic acid and sensitivity to cephalothin, and as Cag A^+ (cytotoxin-associated gene A) and tox^+ (vacuolating cytotoxin) strain (Matsumoto et al 1997). Stock cultures were stored at −80°C in brucella broth (Baltimore Biological Laboratory, Cockeysville, MD) supplemented with 2% heat-inactivated foetal bovine serum.

Infection of mice with H. pylori

Inoculation was performed according to the methods of Karita et al (1991) and Koga et al (1996). Briefly, *H. pylori* was cultured in brucella broth supplemented with 2% foetal bovine serum. The culture was harvested, and the cells were suspended in brucella broth to 2×10^8 colony-forming units mL^{-1}. Mice were inoculated by gavage with bacterial suspension (1·5 mL) 2 days after production of the chronic ulcer. Control (non-infected) animals received vehicle alone.

Measurement of ulcerated area

Animals were killed by inhalation of carbon dioxide 6, 9, 16, 30 or 58 days after gastric ulcer production. The stomachs were removed, inflated with formalin (1%; 1 mL) and immersed in formalin (1%) for 10 min to fix the gastric wall. Subsequently, the stomachs were cut along the greater curvature and the ulcerated area (mm^2) was determined by means of a Luzex F (Nireko, Tokyo, Japan) computer-assisted image analyser system.

In other experiments *H. pylori*-infected mice with gastric ulcer were killed and the number of viable *H. pylori* in the stomach was measured. Whole stomachs were excised using sterile instruments, and the *H. pylori* colonies were counted by procedures previously reported (Koga et al 1996). Briefly, the whole stomachs were homogenized with brucella broth (2 mL) supplemented with 2% heat-inactivated foetal bovine serum, and modified Skirrow's agar plates were inoculated with diluted samples of the homogenate. Plates were incubated at 37°C in a GasPak jar for 5 days in a microaerobic environment and the colonies of *H. pylori* were then counted.

Microscopic examination

Sixteen days after ulcer production the tissue specimens were fixed in Camoy's fixing solution for 2 h, dehydrated with absolute alcohol, processed using standard procedures, embedded in paraffin, and sectioned at 4 μm. Sections were stained by the method of Genta et al (1994) which enables simultaneous visualization of *H. pylori* and gastric morphology. The sections were also stained immunologically with a monoclonal antibody specific to *H. pylori* (Institute of Immunology, Tokyo, Japan).

Effect of metronidazole and amoxicillin on ulcer healing

Either amoxicillin or metronidazole suspended in 0·4% gum tragacanth solution (10 mL kg^{-1}) was given by gavage to mice for 5 consecutive days starting 4 days after the induction of gastric ulcer. *H. pylori* was administered 2 days after ulcer production. The doses of amoxicillin and metronidazole chosen to eradicate *H. pylori* were 10 and 62·4 mg kg^{-1} daily, respectively, both administered orally. Eighteen hours after the final administration of the antibacterial drugs, the mice were killed and the stomachs were examined for ulcer area as described above.

Statistics

Data are presented as the mean ± s.e. per group. Differences between the experimental groups were determined by use of the two-tailed Dunnett's multiple comparison test; and values of $P < 0.05$ were regarded as indicative of significance.

Results

Healing of gastric ulcer

Acetic acid induced the formation of a round solitary mucosal ulcer between the gastric antrum and corpus of each mouse (Figure 1). In non-infected mice these ulcers healed spontaneously, healing being almost complete 58 days after ulcer production (Table 1). Infection with *H. pylori* delayed the healing of these ulcers; the mean sizes of the ulcers 9 and 16 days after ulcer induction were $7{\cdot}44 \pm 1{\cdot}92$ and $3{\cdot}67 \pm 0{\cdot}61\ mm^2$, respectively, 258% and 267% greater than those observed in control mice ($P = 0{\cdot}017$ and $0{\cdot}000018$).

Histopathology

The histology of a typical acetic acid-induced gastric ulcer in *H. pylori*-infected mice 16 days after ulcer induction is shown in Figure 2B. This ulcer was wide open with no re-epithelization at the edge of the ulcer. The ulcer in the non-infected mouse was small, indicating that some healing had occurred (Figure 2A). Multiple staining of the gastric mucosa clearly showed the presence of *H. pylori*; curved, rod-shaped bacteria measuring 4–6 μm were observed by light microscopy (Figures 3A and 4A). Immunohistochemical staining using a monoclonal antibody confirmed the organism as *H. pylori* (Figures 3B and 4B). High densities of *H. pylori* were present in the mucus on the surface of superficial epithelial cells (Figures 3A, B) and also in debris on the base of the ulcer (Figures 4A, B), and some were also found in both healthy and damaged mucosa. Infiltration of inflammatory cells was seen near *H. pylori* colonization in the base of the ulcer (Figures 4A, B).

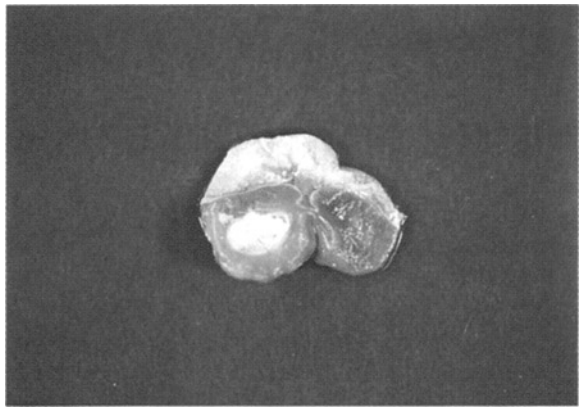

Figure 1. Gastric ulcer in mouse 9 days after induction with acetic acid.

Table 1. Healing of gastric ulcer in *H. pylori*-infected and non-infected mice.

Days after induction of ulcer	Ulcerated area (mm^2)†	
	Non-infected	Infected
6	$13{\cdot}15 \pm 2{\cdot}52$ (12)	$15{\cdot}53 \pm 2{\cdot}40$ (12)
9	$2{\cdot}08 \pm 0{\cdot}50$ (12)	$7{\cdot}44 \pm 1{\cdot}92$ (13)*
16	$1{\cdot}00 \pm 0{\cdot}24$ (10)	$3{\cdot}67 \pm 0{\cdot}61$ (17)**
30	$0{\cdot}01 \pm 0{\cdot}01$ (4)	$1{\cdot}36 \pm 1{\cdot}01$ (5)
58	$0{\cdot}19 \pm 0{\cdot}17$ (4)	$0{\cdot}56 \pm 0{\cdot}49$ (5)

†Values are means ± s.e. (the number of mice is given in parentheses). *$P < 0{\cdot}02$, **$P < 0{\cdot}001$, significantly different from result from non-infected group.

Effect of amoxicillin or metronidazole on the healing of gastric ulcers

Eradication of *H. pylori* with amoxicillin promoted the healing of gastric ulcers in *H. pylori*-infected mice (Table 2). The mean ulcer size was $4{\cdot}07 \pm 0{\cdot}61\ mm^2$ in amoxicillin-treated mice and $13{\cdot}69 \pm 1{\cdot}67\ mm^2$ in control mice ($P = 0{\cdot}000093$). No *H. pylori* were detected in the stomachs of treated mice ($< 10^2$ colony-forming units/stomach, $n = 5$). Metronidazole similarly eradicated *H. pylori* infection ($< 10^2$ colony-forming units/stomach, $n = 5$), but the healing of gastric ulcer was similar to that in the control group. The mean ulcer size was $14{\cdot}27 \pm 3{\cdot}26\ mm^2$ in metronidazole-treated mice and $13{\cdot}69 \pm 1{\cdot}67\ mm^2$ in control mice ($P = 0{\cdot}87$).

Discussion

Infection of germ-free athymic mice with *H. pylori* was developed by Karita et al (1991, 1994) who found that the bacteria persist in the stomach for more than 20 weeks. Development of gastritis and duodenitis in this mouse model after *H. pylori* colonization might be suitable for studying the clinical significance of *H. pylori*-infection (Karita et al 1991; Marchetti et al 1995). Although combination of antibacterial drugs and an anti-ulcer agent cured the *H. pylori* infection in this animal model (Karita et al 1993), the relationship between

Table 2. Effect of antibacterial drugs on healing of gastric ulcer in *H. pylori*-infected mice.

Treatment	Ulcerated area (mm^2)†
Control (*H. pylori*-infected)	$13{\cdot}69 \pm 1{\cdot}67$ (12)
Non-infected	$9{\cdot}22 \pm 1{\cdot}35$ (10)*
Metronidazole (*H. pylori*-infected)	$14{\cdot}27 \pm 3{\cdot}26$ (13)
Amoxicillin (*H. pylori*-infected)	$4{\cdot}07 \pm 0{\cdot}61$ (12)**

†Values are means ± s.e. (the number of mice is given in parentheses). *$P < 0{\cdot}02$, **$P < 0{\cdot}001$, significantly different from result for control group.

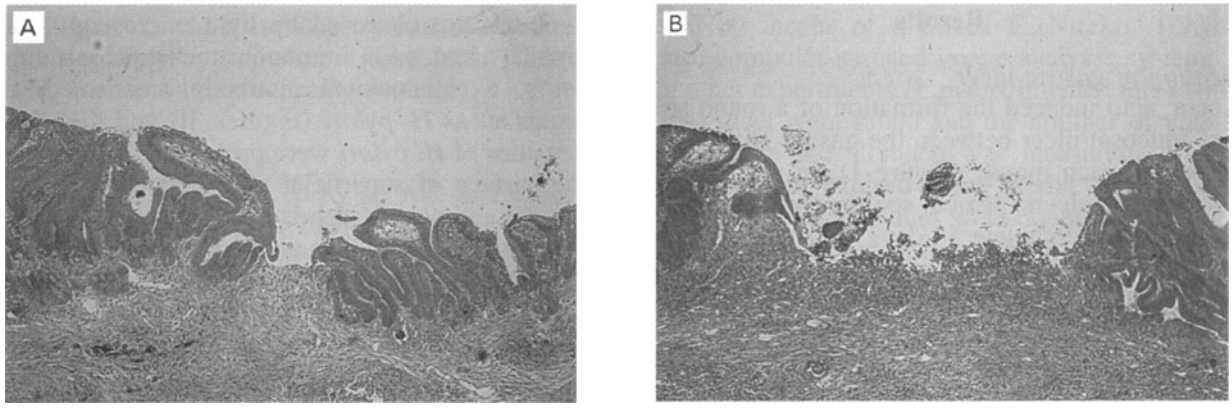

Figure 2. Multiple staining of the ulcerated area 16 days after induction in (A) non-infected mouse stomach and (B) *H. pylori*-infected mouse stomach (magnification × 74).

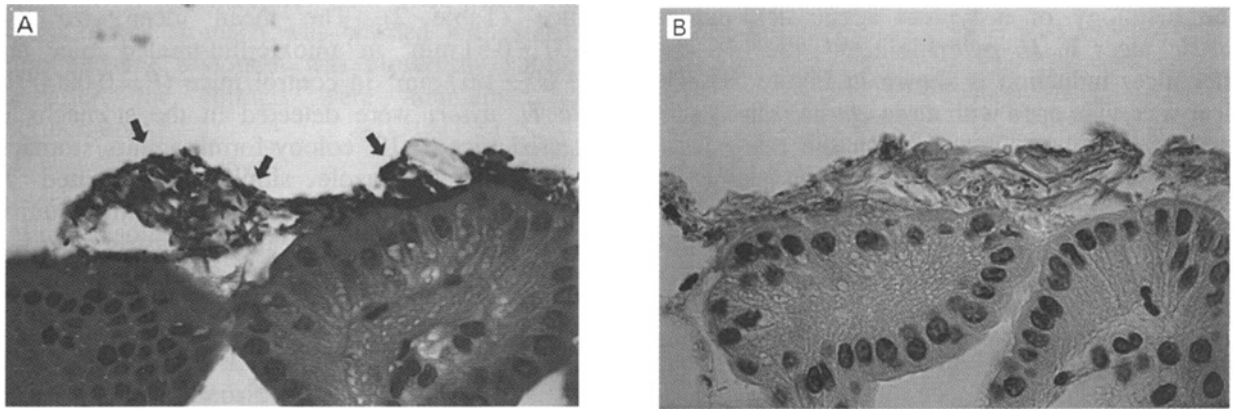

Figure 3. A. Multiple staining of the surface of the gastric mucosa 16 days after ulcer induction in an *H. pylori*-infected mouse stomach (*H. pylori* are indicated by arrows) and B. immunohistochemical identification of *H. pylori* on the surface of the gastric mucosa (magnification × 730).

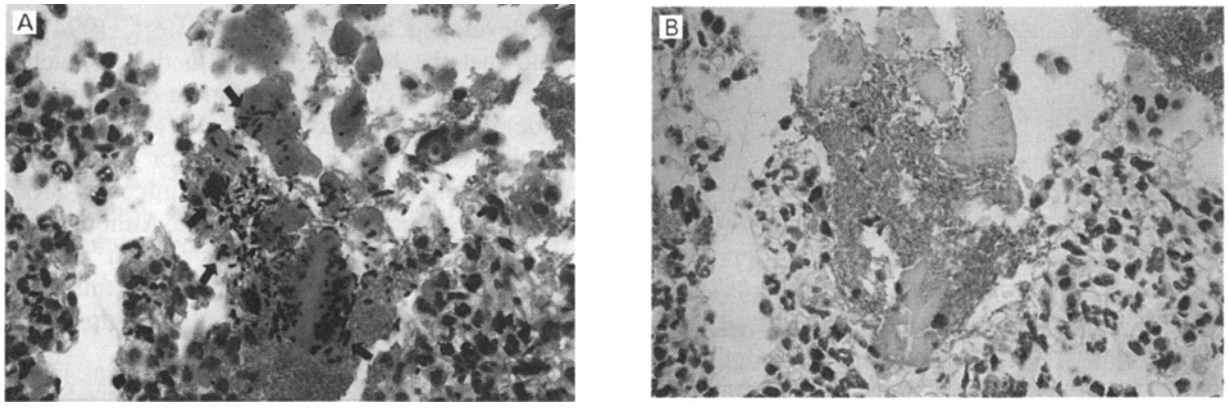

Figure 4. A. Multiple staining of debris at the base of a gastric ulcer 16 days after ulcer induction in an *H. pylori*-infected mouse stomach (*H. pylori* are indicated by arrows) and B. immunohistochemical identification of *H. pylori* in the debris at the base of the ulcer (magnification × 730).

H. pylori infection and the healing of chronic gastric ulcer has not been clearly demonstrated in the animal models. In the current study we have shown for the first time that gastric ulcer healing was significantly delayed by the continuous presence of *H. pylori*. Ross et al (1992) showed that daily gastric administration of *H. pylori* or bacterium-free filtrates caused prolongation of pre-existing gastric ulceration in rats and that *H. pylori* alone had no effect on the normal gastric mucosa of rats, although infection with *H. pylori* was not successful in their study.

H. pylori can induce the production of cytokines (Noach et al 1994; Crowe et al 1995) which are causal factors in mucosal inflammation. This suggests that *H. pylori* might aggravate or prolong the inflammation of gastric mucosa and delay ulcer healing, a contention consistent with the infiltration of inflammatory cells around the organisms in the base of the ulcer. Alternatively, the growth or integrity of gastric epithelial cells might be impaired by *H. pylori* infection. Monochloramine (Dekigai et al 1995; Murakami et al 1995) and cytotoxins (Cover et al 1991; Ghiara et al 1995) from *H. pylori* can damage gastric mucosal cells and might delay gastric ulcer healing.

H. pylori can survive persistently in the athymic mouse stomach. The organisms were clearly present in wide areas of the gastric mucosa, mostly in the gastric mucous layer on the surface epithelial cells and in the ulcer bed. These results accord with the observation of *H. pylori* in the surface mucous layer in man (Shimizu et al 1996).

Clinical studies (Marshall et al 1988; Hentschel et al 1990) indicate that eradication of *H. pylori* can be achieved by use of antibacterial drugs and an anti-ulcer agent, and that this reduces the recurrence of gastric and duodenal ulcers. Amoxicillin and metronidazole can cure the infection in *H. pylori*-infected mice (Karita et al 1993). In the current study we found that amoxicillin counteracted the deleterious influence of *H. pylori* on gastric ulcer healing. These results suggest that *H. pylori* might be a deleterious factor in gastric ulcer healing and that eradication of this organism might accelerate the healing of gastric ulcers. Because amoxicillin can protect the gastric mucosa in rats (Lam et al 1994), this action and its antibiotic effect might also contribute to ulcer healing. It is possible that metronidazole might have a noxious effect on ulcer healing irrespective of its antibacterial effect. Further studies are needed to clarify the different effects of amoxicillin and metronidazole on ulcer healing in this mouse model.

In conclusion, this study has clearly demonstrated that *H. pylori* infection delayed the healing of gastric ulcers induced by the serosal application of acetic acid in mice, possibly by aggravation or prolongation of mucosal inflammation. Amoxicillin, which eradicated the *H. pylori*, promoted gastric ulcer healing in mice previously infected with *H. pylori*.

Acknowledgements

We would like to thank Yuka Inomata and Yukiyo Murakami for their technical assistance, Dr Koji Takeuchi, Department of Pharmacology and Experimental Therapeutics, Kyoto Pharmaceutical College, Kyoto, for his critical reading of this manuscript and Dr Mikio Karita for providing *H. pylori* strain CPY 2052.

References

Baskerville, A., Newell, D. G. (1988) Naturally occurring chronic gastritis and *C. pylori* infection in the rhesus monkey; a potential model for gastritis in man. Gut 29: 465–472

Cover, T. L., Puryear, W., Perez-Perez, G. I., Blaser, M. J. (1991) Effect of urease on HeLa cell vacuolation induced by *Helicobacter pylori* cytotoxin. Infect. Immun. 59: 1264–1270

Crowe, S. E., Alvarez, L., Dytoc, M., Hunt, R. H., Muller, M., Sherman, P., Patel, J., Jin, Y., Ernst, P. B. (1995) Expression of interleukin 8 and CD54 by human gastric epithelium after *Helicobacter pylori* infection in vitro. Gastroenterology 108: 65–74

Dekigai, H., Murakami, M., Kita, T. (1995) Mechanism of *Helicobacter pylori*-associated gastric mucosal injury. Dig. Dis. Sci. 40: 1332–1339

Engstrand, L., Gustavsson, S., Jorgensen, A., Schwan, A., Scheynius, A. (1990) Inoculation of barrier born pigs with *Helicobacter pylori*: a useful animal model for gastritis type B. Infect. Immun. 58: 1763–1768

Fujioka, T., Kubota, T., Shuto, R., Kodama, R., Murakami, K., Perparim, K., Nasu, M. (1994) Establishment of an animal model for chronic gastritis with *Helicobacter pylori*; potential model for long-term observations. Eur. J. Gastroenterol. Hepatol. (Suppl. 1): S73–S78

Genta, R. M., Robason, G. O., Graham, D. Y. (1994) Simultaneous visualization of *Helicobacter pylori* and gastric morphology: a new stain. Human Pathol. 25: 221–225

Ghiara, P., Marchetti, M., Blaser, M. J., Tummuru, M. K., Cover, T. L., Segal, E. D., Tompkins, L. S., Rappuoli, R. (1995) Role of the *Helicobacter pylori* virulence factors vacuolating cytotoxin, CagA, and urease in a mouse model of disease. Infect. Immun. 63: 4154–4160

Graham, D. Y., Klein, P. D., Evans, D. J., Evans, D. G., Alpert, L. C., Opekun, A. R., Boutton, T. W. (1987) *Campylobacter pylori* detected non-invasively by the [^{13}C]urea breath test. Lancet 1: 1174–1177

Graham, D. Y., Lew, G. M., Klein, P. D., Evans, D. G., Evans, D. J. Jr, Saeed, Z. A., Malaty, H. M. (1992) Effect of treatment of *Helicobacter pylori* infection on the long-term recurrence of gastric or duodenal ulcer: a randomized, controlled study. Ann Intern. Med. 116: 705–708

Hentschel, E., Brandstatter, G., Dragosics, B., Hirschl, A. M., Nemec, H., Schutze, K., Tauter, M., Wurzer, H. (1990) Effect of ranitidine and amoxicillin plus metronidazole on the eradication of *Helicobacter pylori* and the recurrence of duodenal ulcer. N. Engl. J. Med. 328: 308–312

Karita, M., Kouchiyama, T., Okita, K., Nakazawa, T. (1991) New small animal model for human gastric *Helicobacter pylori* infection: success in both nude and euthymic mice. Am. J. Gastroenterol. 86: 1596–1603

Karita, M., Li, Q., Okita, K. (1993) Evaluation of new therapy for eradication of *H. pylori* infection in nude mouse model. Am. J. Gastroenterol. 88: 1366–1372

Karita, M., Li, Q., Cantero, D., Okita, K. (1994) Establishment of a small animal model for human *Helicobacter pylori* infection using germ-free mouse. Am. J. Gastroenterol. 89: 208–213

Koga, T., Kawada, H., Utsui, Y., Domon, H., Ishii, C., Yasuda, H. (1996) In-vitro and in-vivo antibacterial activity of plaunotol, a cytoprotective antiulcer agent, against *Helicobacter pylori*. J. Antimicrob. Chemother. 37: 919–929

Krakowka, S., Morgan, D. R., Kraft, W. G., Leunk, R. D. (1987) Establishment of gastric *Campylobacter pylori* infection in the neonatal gnotobiotic piglet. Infect. Immun. 55: 2789–2796

Lam, S. K., Cho, C. H., Chen, B. W., Lai, K. C., Ching, C. K., Ho, C. S., Li, Y. N. (1994) Gastric cytoprotection by amoxicillin in the rat. J. Gastroenterol. Hepatol. 9: 514–518

Marchetti, M., Arico, B., Burroni, D., Figura, N., Rappuoli, R., Ghiara, P. (1995) Development of mouse model of *Helicobacter pylori* infection that mimics human disease [see comments]. Science 267: 1655–1658

Marshall, B. J., Langton, S. R. (1986) Urea hydrolysis in patients with *Campylobacter pyloridis* infection. Lancet 1: 965–966

Marshall, B. J., Goodwin, C. S., Warren, J. R., Murray, R., Blincow, E. D., Blackbourn, S. J., Phillips, M., Waters, T. E., Sanderson, C. R. (1988) Prospective double-blind trial of duodenal ulcer relapse after eradication of *Campylobacter pylori*. Lancet 2: 1437–1442

Matsumoto, S., Washizuka, Y., Matsumoto, Y., Tawara, S., Ikeda, Y., Yokota, Y., Karita, M. (1997) Induction of ulceration and severe gastritis in Mongolian gerbil by *Helicobacter pylori* infection. J. Med. Microbiol. 46: 391–397

Murakami, M., Asagoe, K., Dekigai, H., Kusaka, S., Saita, H., Kita, T. (1995) Products of neutrophil metabolism increase ammonia-induced gastric mucosal damage. Dig. Dis. Sci. 40: 268–273

Noach, L. A., Bosma, N. B., Jansen, J., Hock, F. J., van Deventer, S. J., Tytgat, G. N. (1994) Mucosal tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 production in patients with *Helicobacter pylori* infection. Scand. J. Gastroenterol. 29: 425–429

Perterson, W. L. (1991) *Helicobacter pylori* and peptic ulcer disease. N. Engl. J. Med. 324: 1043–1048

Ross, J. S., Bui, H. X., del Rosario, A., Sonbati, H., George, M., Lee, C. Y. (1992) *Helicobacter pylori*. Its role in the pathogenesis of peptic ulcer disease in a new animal model. Am. J. Pathol. 141: 721–727

Shimizu, T., Akamatsu, T., Ohta, T., Katsuyama, T. (1996) Immunohistochemical detection of *Helicobacter pylori* in the surface mucous gel layer and its clinicopathological significance. Helicobacter 1: 197–206

Shuto, R., Fujioka, T., Kubota, T., Nasu, M. (1993) Experimental gastritis induced by *Helicobacter pylori* in Japanese monkeys. Infect. Immun. 61: 933–939

Takagi, K., Okabe, S., Saziki, R. (1969) A new method for the production of chronic gastric ulcer in rats and effect of several drugs on its healing. Jpn J. Pharmacol. 19: 418–426

Warren, J. R., Marshall, B. J. (1983) Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet 1: 1273–1275