

## Reversibility of Heterotopic Proliferative Glands in Glandular Stomach of *Helicobacter pylori*-infected Mongolian Gerbils on Eradication

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*Helicobacter pylori* (*Hp*) infection is an important factor in human gastric disorders. Mongolian gerbils can be easily infected with *Hp* and represent excellent experimental models to clarify the role of *Hp* in chronic active gastritis, peptic ulcers, intestinal metaplasia, and gastric carcinoma. We have proved the enhancing effects of *Hp* infection on all histological types of gastric cancers in Mongolian gerbils exposed to chemical carcinogens. Heterotopic proliferative glands (HPGs) also frequently develop with *Hp* infection in the glandular stomach of infected gerbils, with a slightly dysplastic change of constituent cells. Distinguishing reversible inflammatory lesions from true neoplasms upon eradication is necessary for further biological or histochemical investigations using this model. We employed an experimental model of long-term *Hp* infection and eradication in gerbils. HPGs finally developed with a phenotypic shift of intestinalization with Paneth cells. After eradication, HPGs were obviously reduced, and gastric lesions in mucosa also improved with few remnants of the former injury. This shows that reversible HPGs are frequently induced solely by *Hp* infection in this animal species, and are related to severe gastritis, rather than being malignant in character. Thus, distinguishing reversible lesions from true neoplasms is necessary to investigate the relationship of *Hp* infection and gastric carcinogenesis in this animal model.

Key words: *Helicobacter pylori* — Eradication — Gastric carcinogenesis — Intestinal metaplasia — Mongolian gerbils

*Helicobacter pylori* (*Hp*) infection reportedly has a positive correlation with gastric cancer development, based on epidemiological findings in humans.<sup>1–3</sup> In 1994, WHO/IARC thus concluded that *Hp* is a 'definite carcinogen.'<sup>4</sup> Mongolian gerbils can be infected with *Hp*, and the resultant chronic active gastritis, peptic ulcers, and intestinal metaplasia resemble human lesions. This is the rationale for its adoption as an experimental model for detailed analysis of the role of *Hp* in gastric disorders. To assess the putative causal link between *Hp* infection and carcinogenesis in the glandular stomach, we earlier treated gerbils with a combination of chemical carcinogen exposure and *Hp* infection,<sup>5</sup> and proved enhancing effects on tumor development induced by exposure to N-methyl-N-nitrosourea (MNU)<sup>6</sup> or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).<sup>7</sup> We further demonstrated that *Hp* infection enhances genesis of all histological types of stomach cancer, mimicking the human case,<sup>8</sup> and that eradication exerts a suppressive effect.<sup>9</sup>

Submucosal heterotopic proliferative glands also develop in the glandular stomach of gerbils with *Hp* infection alone, often resembling differentiated carcinomas.

Our previous studies from the standpoint of histopathology showed no carcinomas in animals only treated with *Hp* infection.<sup>5–9</sup> However, two reports concluded that *Hp* infection alone can induce well-differentiated adenocarcinomas at very high incidences (37%<sup>10</sup>, 40%<sup>11</sup>) in the glandular stomach of gerbils, while another report found that *Hp* infection induced only one poorly-differentiated adenocarcinoma (1.8%).<sup>12</sup> The incidences and histological patterns of the lesions differed greatly in these three papers. After *Hp* infection, glands in the stomach of Mongolian gerbils start to proliferate into the submucosa, disrupting the lamina muscularis mucosa. No papers have mentioned the histological characteristics and differential diagnosis between these frequent submucosal proliferating glands and well-differentiated adenocarcinomas in this animal species.

Mongolian gerbils are considered to be excellent experimental models to clarify the role of *Hp* in chronic active gastritis, peptic ulcers, intestinal metaplasia, and gastric carcinoma. But submucosal proliferating glands, often resembling adenocarcinomas, also frequently develop in the glandular stomach of *Hp*-infected gerbils, so the establishment of a pathological consensus regarding gastric carcinoma in this animal is required. In the Japanese human population, it is reported that 2–3% of all *Hp*-infected

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people have symptoms of gastric or duodenal ulcers, and that only 0.4% develop gastric carcinomas. These figures have significant implications for the role of chronic *Hp* infection.

In the present study, we defined the submucosal heterotopic proliferating structure-altered glands in the glandular stomach of gerbils, as heterotopic proliferative glands (HPGs). We infected gerbils with *Hp*, and performed eradication long after the infection. HPGs, developing in the glandular stomach of *Hp*-infected gerbils, were examined in terms of histochemical phenotypes.

## MATERIALS AND METHODS

*Hp* samples containing about  $1.0 \times 10^8$  colony-forming units (0.8 ml) per milliliter were used as the inoculum, delivered intra-gastrically via an oral catheter after fasting for 24 h. For eradication of *Hp*, a 'triple therapy' was performed. Lansoprazole, amoxicillin and clarithromycin, were suspended in 0.5% wt/wt carboxymethyl cellulose sodium salt solution and administered intra-gastrically twice a day for two successive days at doses of 10, 3 and 30 mg per kg body weight, respectively. The experimental design, described below, was approved by the Animal Care Committee of the Aichi Cancer Center Research Institute. Specific-pathogen-free, male, 7-week-old, Mongolian gerbils (*Meriones unguiculatus*, MGS/Sea, Seac Yoshitomi, Ltd., Fukuoka), were housed in steel cages on hard wood chip bedding in an air-conditioned biohazard room for infection, with a 12 h light-12 h dark cycle. They were given food (Oriental MF, Oriental Yeast Co., Tokyo) irradiated with 30 kilo-Gy gamma rays and autoclaved distilled water *ad libitum*.

Animals in groups 1 and 2 received no treatment and were sacrificed after 50 and 100 weeks as controls, respectively. Animals in groups 3–6 received *Hp* and were sacrificed at 25, 50, 75, and 100 weeks after infection, respectively. Animals in group 7 received *Hp*, underwent eradication at 75 weeks after infection, and were sacrificed 25 weeks thereafter. Animals in group 8 received *Hp*, underwent eradication at week 50, and were sacrificed at week 100 (Fig. 1). All animals were injected with 100 mg/kg BW of 5-bromo-2'-deoxyuridine (BrdU) intra-peritoneally, and 1 h later they were subjected to deep ether anesthesia, and then laparotomized with excision of their stomachs. Tissues were processed for microbiological and histopathological examination. The excised stomachs were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.2) or 95% ethanol containing 1% acetic acid, cut into about 16 strips, processed by standard methods and embedded in paraffin. Tissues were sectioned at 5  $\mu$ m for staining with hematoxylin and eosin (H&E), alcian blue-periodic acid Schiff (AB-PAS), paradoxical concanavalin A (PCA), and immunohistochemistry for BrdU

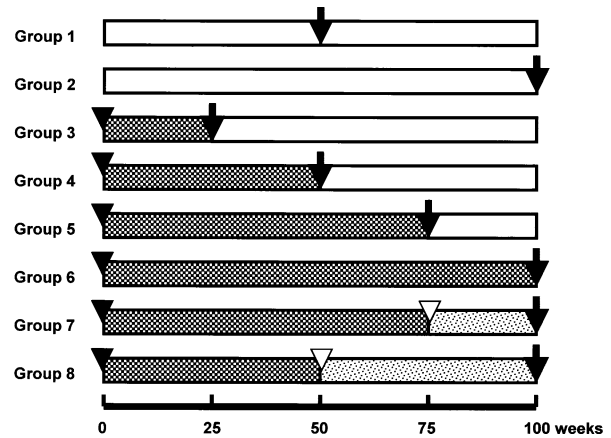


Fig. 1. Experimental design: Gerbils with no treatment were sacrificed at 50 (group 1:  $n=9$ ) and 100 (group 2:  $n=4$ ) weeks. Gerbils infected with *Hp* were examined after periods of 25 (group 3:  $n=10$ ), 50 (group 4:  $n=18$ ), 75 (group 5:  $n=13$ ), and 100 (group 6:  $n=21$ ) weeks. Eradication was also performed for two groups at 50 (group 7:  $n=13$ ) and 75 (group 8:  $n=16$ ) weeks, with sacrifice at 100 weeks. ▼: inoculation of *Hp*, ▽: eradication, ↓: sacrifice.

and *Hp* (anti-*Hp* serum, DAKO, Glostrup, Denmark). Areas of HPGs and withering of parietal cells were assessed using a micrometer, counting areas of 0.015625 mm<sup>2</sup> ( $\times 40$ ; HPGs), or 0.0625 mm<sup>2</sup> ( $\times 20$ ; areas of fundic glands with >50% of parietal cells). Areas per length of slices of glandular stomach (mm<sup>2</sup>/cm) were then calculated. Mann-Whitney's *U* test was applied to establish the significance of differences in areas of HPGs and withering of parietal cells. *P* values less than 0.05 were considered to be statistically significant.

## RESULTS

In control animals (groups 1 and 2), no histological changes were observed in either pyloric or fundic glands.

After infection with *Hp*, the glandular stomach epithelium showed hyperplastic changes, with varying degrees of multifocal cystic dilatation and erosion, as well as marked infiltration, predominantly of lymphocytes and some macrophages and neutrophils, in the lamina propria and submucosa. Throughout the study, immunohistochemical staining for *Hp* allowed the observation of bacteria in *Hp*-infected and non-eradicated animals (groups 3–6).

At 25 weeks after *Hp* infection (group 3), gastric glands in the border area proliferated into submucosa with interruptions of the lamina muscularis mucosa (Fig. 2A). These submucosal heterotopic proliferating glands, surrounded with collagen fibers, produced gastric mucin, stained yellow with PCA (Fig. 2B), and were classified as gastric-

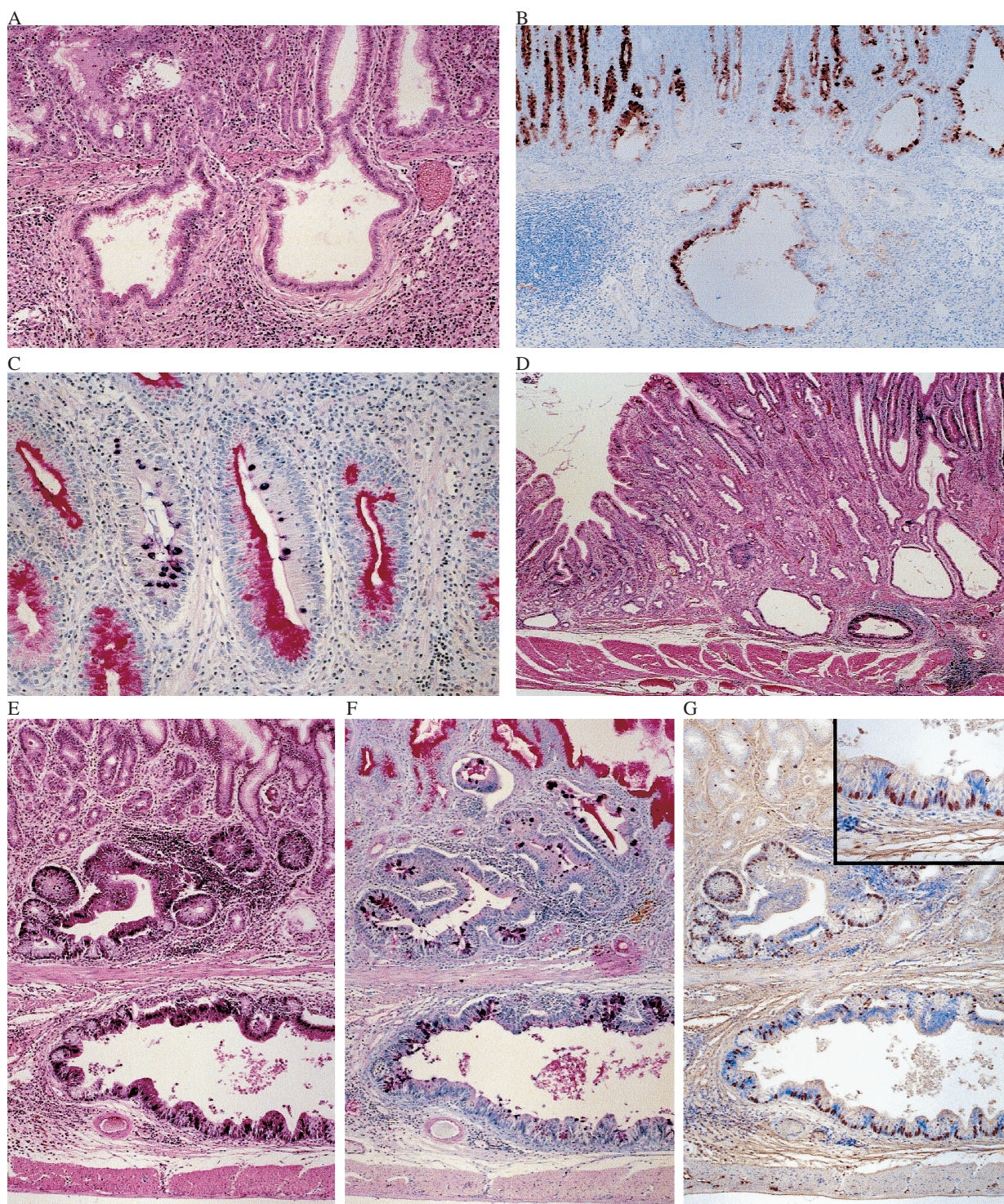


Fig. 2. A. Penetrating HPGs, surrounded with collagen fibers. Group 3 ( $\times 25$ : H&E). B. Gastric-type HPGs. Group 3 ( $\times 25$ : PCA). C. GI mixed intestinal metaplasia. Group 4 ( $\times 50$ : AB-PAS). D. Hyperplastic changes with multifocal cystic dilatation, infiltration of inflammatory cells, intestinal metaplasia and HPGs. Group 4 ( $\times 10$ : H&E). E. Submucosal HPG under intestinal metaplasia. Group 4 ( $\times 25$ : H&E). F. I-type HPG under intestinal metaplasia. Group 4 ( $\times 25$ : AB-PAS). G. Intestinal crypt organoid structures with a proliferating zone at the base. Group 4 ( $\times 25$ : BrdU).

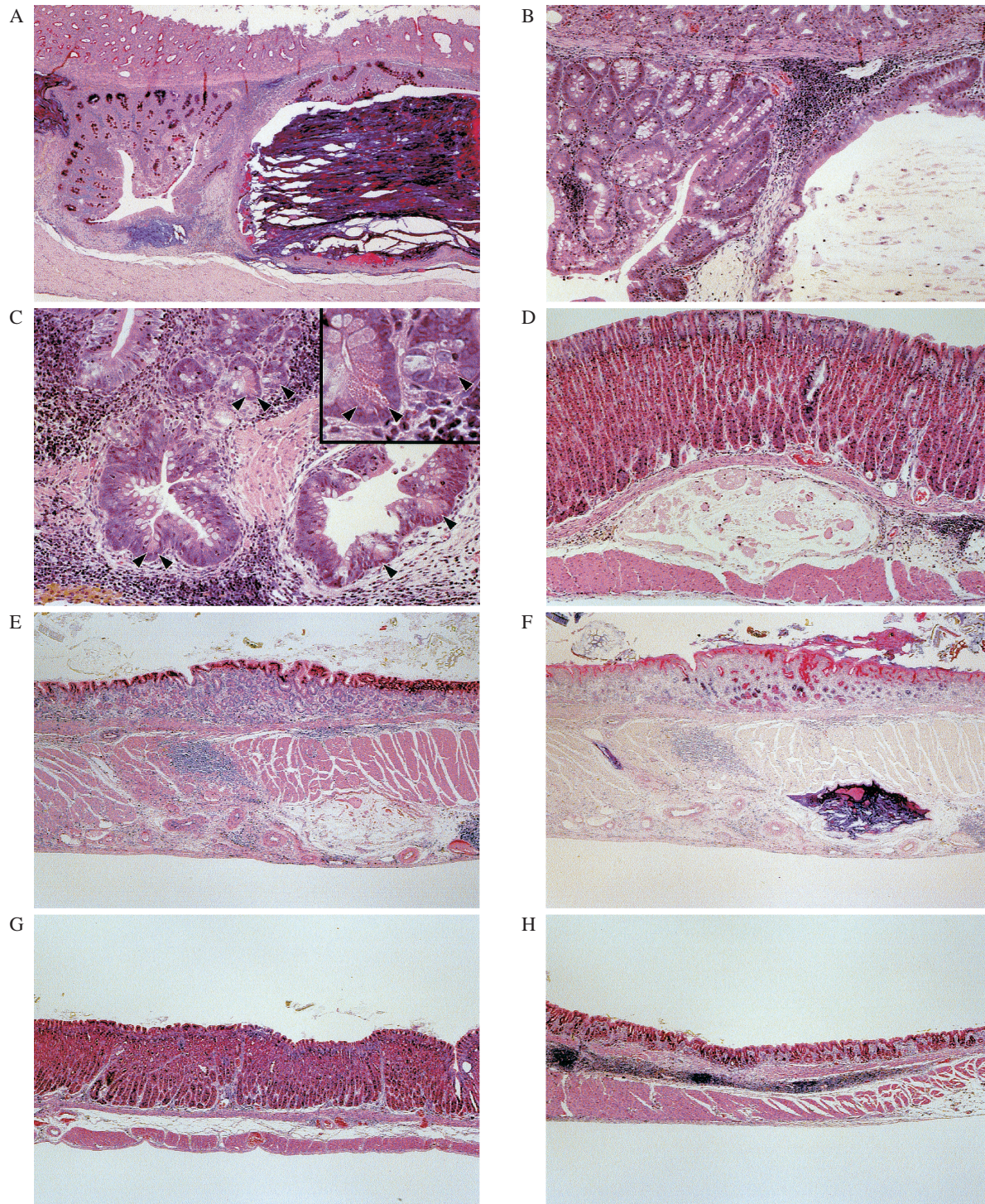


Fig. 3. A. I-type HPGs, with cystic dilatation containing mucin. Group 4 ( $\times 16$ : AB-PAS). B. I-type HPGs under the lamina muscularis mucosa. Group 4 ( $\times 40$ : H&E). C. I-type HPGs with Paneth cells (arrowheads). Group 5 ( $\times 50$  [inset  $\times 100$ ]: H&E). D. Remnant submucosal mucin lake without epithelial cells, under repaired fundic glands. Group 7 ( $\times 25$ : H&E). E. Remnant subserosal mucin lake, under the muscularis propria. Group 7 ( $\times 16$ : H&E). F. Remnant subserosal mucin lake stained with AB-PAS. Group 7 ( $\times 16$ : AB-PAS). G. Repaired fundic glands with some remnants of fibrosis. Group 8 ( $\times 10$ : H&E). H. Partial interruptions of the lamina muscularis mucosa, fibrosis and remnant of gathering of inflammatory cells in the pyloric area. Group 7 ( $\times 10$ : H&E).

type HPGs (G-type HPGs). These structure-altered proliferating glands were seen in all animals in group 3. A few foci of intestinal metaplasia were seen in the pyloric areas of the stomach.

Fifty weeks after *Hp* infection, intestinalization was observed both in mucosal glands and submucosal HPGs (group 4). In some metaplastic glands, half of the cells had been replaced by intestinal-type cells consisting of intestinal absorptive cells with striated cell borders and goblet cells with mucin stained blue with AB-PAS, and the other half remained gastric-type cells (Fig. 2C), thus presenting as GI mixed intestinal metaplasia. The submucosal HPGs also showed gradual intestinalization with phenotypic shift from G-type to GI mixed-type, and were thus classified as GI mixed-type HPGs. Fundic glands lost their parietal cells with progression of inflammation and mucosal injury,

represented by hyperplastic changes with varying degrees of multifocal cystic dilatation, erosion, infiltration of inflammatory cells and intestinal metaplasia (Fig. 2D).

GI mixed-type HPGs finally showed a phenotypic shift to intestinal type, thus presenting as I-type HPGs (Fig. 2, E and F). I-type HPGs demonstrated intestinal crypt organoid structures with polarity of proliferating zone at the base, revealed by BrdU staining (Fig. 2G).

Most HPGs showed intestinal type at 75 weeks after infection (group 5) (Fig. 3, A and B). They consisted of intestinal-type cells, while gastric-type cells were decreased. Mature intestinal-type HPGs with Paneth cells could also be seen at 75 or 100 weeks after infection (groups 5 and 6), thus presenting as I-type HPGs with Paneth cells (Fig. 3C). I-type HPGs were seen in all (100%) long-infected gerbils (groups 5 and 6) in this study. The exis-

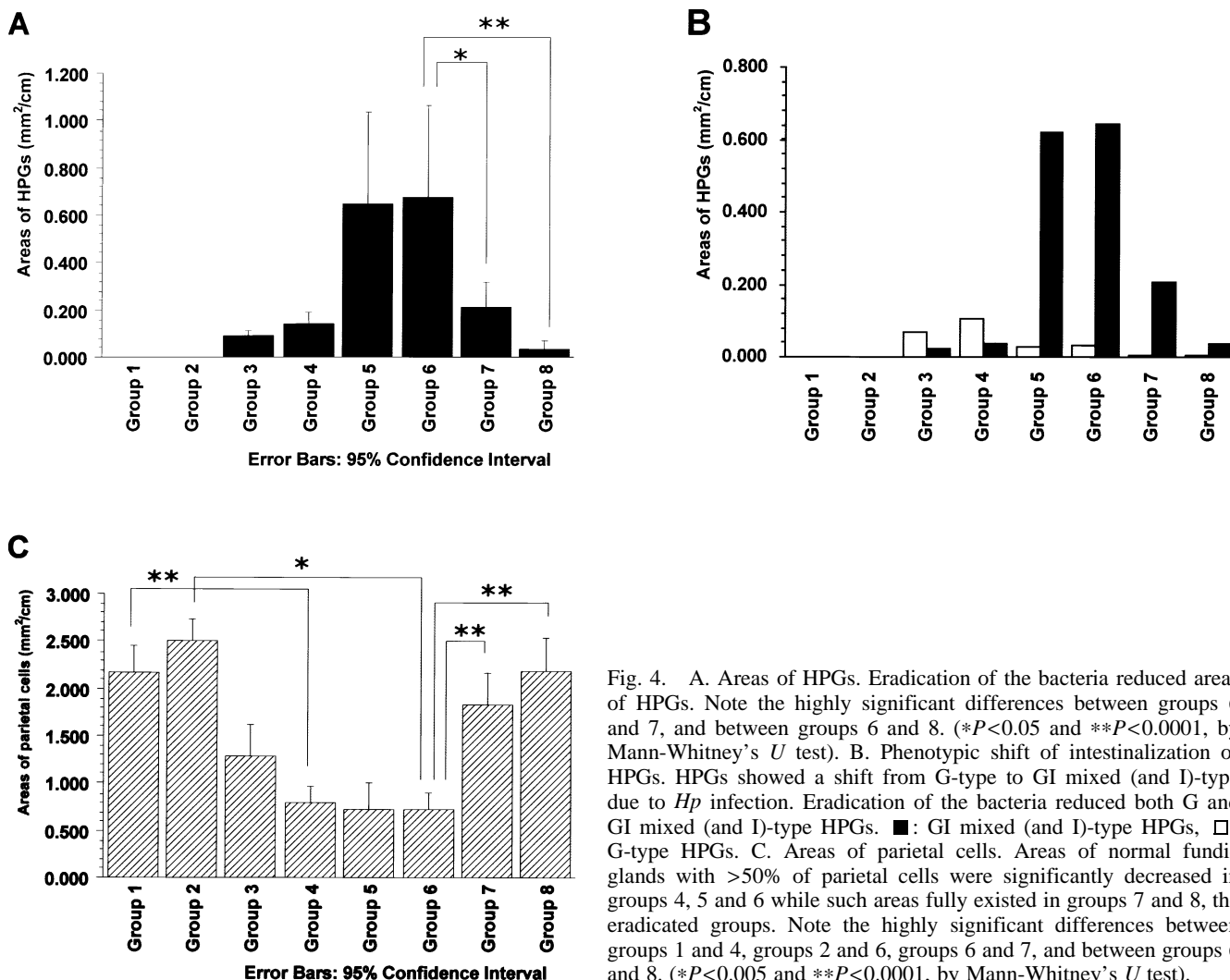


Fig. 4. A. Areas of HPGs. Eradication of the bacteria reduced areas of HPGs. Note the highly significant differences between groups 6 and 7, and between groups 6 and 8. (\* $P < 0.05$  and \*\* $P < 0.0001$ , by Mann-Whitney's  $U$  test). B. Phenotypic shift of intestinalization of HPGs. HPGs showed a shift from G-type to GI mixed (and I)-type due to *Hp* infection. Eradication of the bacteria reduced both G and GI mixed (and I)-type HPGs. ■: GI mixed (and I)-type HPGs, □: G-type HPGs. C. Areas of parietal cells. Areas of normal fundic glands with  $>50\%$  of parietal cells were significantly decreased in groups 4, 5 and 6 while such areas fully existed in groups 7 and 8, the eradicated groups. Note the highly significant differences between groups 1 and 4, groups 2 and 6, groups 6 and 7, and between groups 6 and 8. (\* $P < 0.005$  and \*\* $P < 0.0001$ , by Mann-Whitney's  $U$  test).

tence of Paneth cells was considered to represent the maintained regularity of differentiation with the phenotypic shift of intestinalization.

HPGs showed a shift from G-type to GI mixed-type or I-type with the appearance of Paneth cells during the overall course of *Hp* infection, forming large cystic lesions, surrounded with collagen fibers, containing mucin. They formed mucinous cystic lesions with dysplastic changes, resembling mucinous adenocarcinomas. However, they also showed shedding of their epithelial cells and necrosis at the tips of the lesions.

Eradication at week 75 (group 7) or 50 (group 8) after *Hp* inoculation resulted in reduced HPGs (Fig. 3, D–H). The intestinal metaplasia was also reduced. Fibrosis, partial interruption of the lamina muscularis mucosa, submucosal gathering of lymphocytes or neutrophils, and regenerative changes were occasionally seen as evidence of former injury to the mucosa. Parietal cells, decreased in the time course of inflammation with *Hp* infection, reappeared long after eradication. The intestinal-type mucin without epithelial cells occasionally seen in the muscle layer or subserosa was presumed to be remnants of former submucosal HPGs (Fig. 3, D–F). Partial interruptions of the lamina muscularis mucosa with fibrosis were seen as evidence of former injury in the pyloric mucosa (Fig. 3H).

Data on the areas of HPGs, before and after eradication, and the phenotypic shift of intestinalization in lesions are shown in Fig. 4, A and B. There were highly significant differences in the total areas of HPGs between the eradicated and non-eradicated groups (Fig. 4A). HPGs showed a shift from G-type to GI-type or I-type with the appearance of Paneth cells during the overall course of *Hp* infection (Fig. 4B). Eradication of the bacteria reduced both G and GI mixed (and I)-type HPGs. Data on areas of G-type HPGs in groups 1–8 were 0.000, 0.000, 0.067 [0.040–0.094], 0.106 [0.070–0.141], 0.027 [–0.005–0.059], 0.032 [–0.005–0.068], 0.006 [–0.001–0.013], and 0.005 [–0.001–0.011] (mm<sup>2</sup>/cm) (average [95%CI]), respectively. Data on the areas of GI mixed (and I)-type HPGs in groups 1–8 were 0.000, 0.000, 0.021 [–0.008–0.051], 0.035 [0.004–0.065], 0.622 [0.237–1.008], 0.644 [0.249–1.039], 0.209 [0.106–0.313], and 0.035 [0.004–0.065] (mm<sup>2</sup>/cm) (average [95%CI]), respectively. Data on the total areas of HPGs in groups 1–8 were 0.000, 0.000, 0.088 [0.062–0.115], 0.140 [0.091–0.190], 0.649 [0.263–1.036], 0.676 [0.284–1.068], 0.215 [0.113–0.317], and 0.038 [0.008–0.069] (mm<sup>2</sup>/cm) (average [95%CI]), respectively. There were highly significant differences in area of HPGs between group 6 and group 8 ( $P < 0.0001$ ), and between group 6 and group 7 ( $P < 0.05$ ) by Mann-Whitney's *U* test.

Parietal cells, decreased in the time course of inflammation with *Hp* infection, reappeared long after eradication. Data on the areas of normal fundic glands with >50% of

parietal cells are given in Fig. 4C. Data on the areas in groups 1–8 were 2.169 [1.888–2.451], 2.513 [2.295–2.732], 1.294 [0.962–1.627], 0.790 [0.605–0.976], 0.721 [0.440–1.003], 0.720 [0.523–0.898], 1.836 [1.510–2.162], and 2.181 [1.825–2.537] (mm<sup>2</sup>/cm) (average [95%CI]), respectively. There were highly significant differences between group 1 and group 4 ( $P < 0.0001$ ), group 2 and group 6 ( $P < 0.0005$ ), group 6 and group 7 ( $P < 0.0001$ ), and between group 6 and group 8 ( $P < 0.0001$ ) by Mann-Whitney's *U* test.

## DISCUSSION

Mongolian gerbils can be infected with *Hp*, and the resultant chronic active gastritis, peptic ulcers, and intestinal metaplasia resemble lesions apparent in man. This is the rationale for its adoption as an experimental model for detailed analysis of the role of *Hp* in gastric disorders. We previously documented the promoting effects of *Hp* on gastric carcinogenesis. Submucosal heterotopic proliferative glands also developed in the glandular stomach of gerbils with *Hp* infection alone, but studies concentrating attention on the histopathology standpoint showed no carcinomas in only *Hp*-infected animals.<sup>5–9)</sup>

The characteristics of the HPGs include: 1. organized polarity of their component cells; 2. differentiation from gastric-type into intestinal-type with mature Paneth cells; 3. formation of large cystic dilatation containing mucin, often with calcification; 4. shedding of epithelial cells and necrosis at the tips of lesions; 5. related to high-grade inflammation with infiltration of inflammatory cells; and 6. organized polarity of proliferating zone. These characteristics are quite different from those of well-differentiated adenocarcinomas, with obvious cellular atypia, in Mongolian gerbils observed in our studies.<sup>5–9)</sup>

There have been reports that *Hp* infection alone can induce adenocarcinomas at a low or very high incidence in the glandular stomach of gerbils.<sup>10–12)</sup> The reported incidences and the histological patterns of the lesions differ greatly in the literature, with no mention of diagnosis or the frequency of these submucosal proliferative lesions. The histopathological basis for the diagnosis of the carcinomas in two papers<sup>10, 11)</sup> is rather unclear, while another report<sup>12)</sup> found that *Hp* infection induced only one poorly-differentiated adenocarcinoma (1.8%), with clear cellular atypia. In this study in long-infected gerbils, we frequently found mucinous cystic lesions like those described in the two papers mentioned above.<sup>10, 11)</sup> Although these mucinous cystic lesions resembled adenocarcinomas, with the maintained organized polarity of their component cells, diagnoses of carcinoma were not warranted.

Mongolian gerbils are considered to be excellent experimental models to clarify the role of *Hp* in chronic active gastritis, peptic ulcers, intestinal metaplasia, and gastric

carcinoma. But HPGs also frequently develop in *Hp*-infected gerbils, so further studies are needed using the established pathological consensus regarding gastric carcinoma in this animal species. Distinguishing benign or pre-malignant lesions from true neoplasms is necessary for further investigations using this model.

The present investigation demonstrated the nature of HPGs caused by *Hp* infection in the stomach of Mongolian gerbils. They showed a phenotypic shift in the regularity of intestinalization finally with Paneth cells, which are relatively rare in gastric carcinoma. Further, they were markedly reversible on eradication. This reversibility may not necessarily indicate a non-neoplastic character, but the results are important and can not be ignored. They indicate that most lesions might be regenerative changes induced by inflammation, rather than being malignant in character. The lesions targeted here are characteristic of gerbils, and have no direct equivalent in humans.

Many reports have pointed out complex relationships between *Hp* infection and carcinogenesis in the human stomach. But some studies have cast doubt on the link between *Hp* infection and an elevated risk of stomach cancer.<sup>13, 14)</sup> *Hp* infection almost always results in chronic antral gastritis, but only a small proportion of patients develop stomach cancers. Tomb *et al.*<sup>15)</sup> elucidated the complete *Hp* genome sequence in 1997, and Alm *et al.*<sup>16)</sup> have compared two unrelated isolates. Protein-protein interactions also need more attention.<sup>17)</sup> Some *Hp* strains might be more 'carcinogenic,' but host reactions may also be important. El-Omar *et al.*<sup>18)</sup> showed interleukin 1 (IL-1) polymorphisms to be associated with the risk of gastric cancer. The titers of anti-*Hp* antibodies in tumor-bearing animals were found to be higher than in those of tumor-

free gerbils treated in the same manner in our previous study,<sup>7)</sup> implying that T helper 2 humoral responses may also play an important role in stomach carcinogenesis. Persistent inflammation might also serve to promote carcinogenesis.

To our knowledge, this is the first report concerning the reversibility of submucosal heterotopic proliferating glands penetrating into the glandular stomach of *Hp*-infected Mongolian gerbils. The precise investigation of these lesions, HPGs, including the elucidation of their possible pre-malignant characters, will provide new information concerning gastric carcinogenesis.

Various factors underlying inflammation, cell proliferation, apoptosis and biological mediators after *Hp* infection or eradication, with or without exposure to chemical carcinogens, can be examined using the present model to help elucidate the mechanisms of gastric carcinogenesis and possibly provide a firm basis for human cancer prevention.

#### ACKNOWLEDGMENTS

This work was supported in part by a Research Grant from the Princess Takamatsu Cancer Research Fund (00-23207), a Grant-in-Aid for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid from the Ministry of Education, Culture, Science, Sports and Technology of Japan. Lansoprazole, amoxicillin, and clarithromycin for this study were kindly provided by Takeda Chemical Industries, Ltd., Kyowa Hakko Kogyo Co., Ltd. and Taisho Pharmaceutical Co., Ltd., respectively.

(Received September 7, 2001/Revised November 16, 2001/2nd Revised January 31, 2002/Accepted February 4, 2002)

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