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Effects of *Helicobacter pylori* Infection on the Link between Regenerating Gene Expression and Serum Gastrin Levels in Mongolian Gerbils

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SUMMARY: Although regenerating gene (Reg) protein is reported to have a trophic effect on gastric epithelial cells, its involvement in human gastric diseases is not clear. We have recently shown that both gastrin and gastric mucosal inflammation enhance *Reg* gene expression in the fundic mucosa in rats. This study was designed to clarify whether Reg protein is involved in *Helicobacter pylori*–induced gastritis and whether *Reg* gene expression is linked to serum gastrin levels in this condition. Mongolian gerbils were inoculated with an *H. pylori* strain isolated from a gastric cancer patient. Four weeks later, some of the gerbils with *H. pylori* infection were eradicated by lansoprazole, amoxicillin, and clarithromycin. The time courses of changes in *Reg* gene expression, serum gastrin levels, gastric acidity, and histopathologic factors were examined. Four weeks after *H. pylori* infection, gastritis started spreading to the fundic mucosa, and gastric acidity started reducing. Serum gastrin levels and *Reg* mRNA expression in the fundus were significantly increased 6 weeks after infection. *Reg* mRNA expression in the fundus correlated significantly with both serum gastrin levels and the severity of fundic mucosal inflammation. After *H. pylori* eradication, serum gastrin levels and fundic mucosal inflammation were normalized, and the increase in *Reg* mRNA expression was abolished. The *Reg* gene is associated with hypergastrinemia and fundic mucosal inflammation and may be involved in *H. pylori*–induced gastritis. (*Lab Invest 2003, 83:1777–1786*).

The regenerating gene (*Reg*) was isolated from a cDNA library derived from regenerating rat pancreatic islets (Terazono et al, 1988). Thereafter, Reg protein was found to be identical to pancreatic stone protein, lithostathine, and pancreatic thread protein, all of which had been discovered independently and were given several different names (De Caro et al, 1987; Giorgi et al, 1989; Rouquier et al, 1989). Subsequently, Watanabe et al (1994) found that Reg protein is mitogenic to islet cells and suggested that it may be an endogenous growth factor in pancreatic islets. Recently, we showed that Reg protein is overexpressed in enterochromaffin-like (ECL) cells during the healing of gastric ulcer lesions (Asahara et al, 1996; Kawanami et al, 1997) and suggested that it has a trophic effect on gastric epithelial cells (Fukui et al, 1998). However, the involvement of Reg protein in gastric disease remains unclear.

Helicobacter pylori has been identified as a major pathogen involved in the development of chronic gastritis and gastroduodenal ulcer disease as well as gastric adenocarcinoma (Marshall and Warren, 1984; Parsonnet et al, 1991). Many investigators have carried out intensive studies on the pathophysiology of *H. pylori*–induced gastritis, as this may lead to gastric ulcer and adenocarcinoma. *H. pylori* infection is known to induce various biological and immunological responses in the stomach. For example, serum levels of gastrin, which regulate gastric acid secretion and have a trophic effect on gastric ECL cells (Hersey and Sachs 1995; Kinoshita et al, 1998), are elevated in patients with chronic gastritis induced by *H. pylori* infection (Calam, 1996; McGowan et al, 1996). We have previously shown that gastrin acts as an endogenous stimulant for *Reg* expression in ECL cells (Fukui et al, 1998). In addition, we and other investigators have shown that some cytokines also enhance *Reg* expression in vivo and in vitro (Akiyama et al, 2001; Dusetti et al, 1996; Kazumori et al, 2000). These data suggest that Reg may be involved in the pathophysi-

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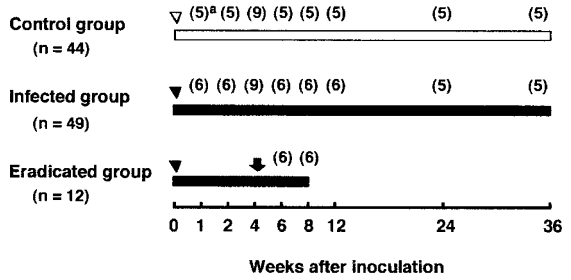

Figure 1.

Schedule of the study. Mongolian gerbils received either brain-heart infusion broth containing *Helicobacter pylori* (*black triangles*) or brain-heart infusion broth alone (*white triangle*). A subgroup of the infected gerbils underwent *H. pylori* eradication using lansoprazole, amoxicillin, and clarithromycin (*black arrow*). [a]Number of animals in each group examined at each time point.

ology of *H. pylori*–induced gastritis. Accordingly, the present study was designed to investigate the effect of *H. pylori* infection on *Reg* expression in Mongolian gerbils and to elucidate the role of *Reg* in the development of gastritis.

Results

Effect of H. pylori *Infection on Histopathologic Findings*

The experimental schedules, including the number of gerbils in each group killed at each time point, are summarized in Figure 1. The uninfected control animals showed no histopathologic abnormalities throughout the observation period. The histopathologic changes occurring in the gastric antrum and corpus of *H. pylori*–infected gerbils for up to 36 weeks are shown in Figure 2.

Neutrophils and Mononuclear Cells

At 1 week after *H. pylori* inoculation, the gastric mucosa showed almost no histologic changes. At 2 weeks after inoculation, mild inflammatory cell infiltration became apparent in the lamina propria of the antrum, especially in the area close to the pyloric ring (Figs. 2 and 3, A and B); however, no remarkable infiltration of inflammatory cells was observed in the

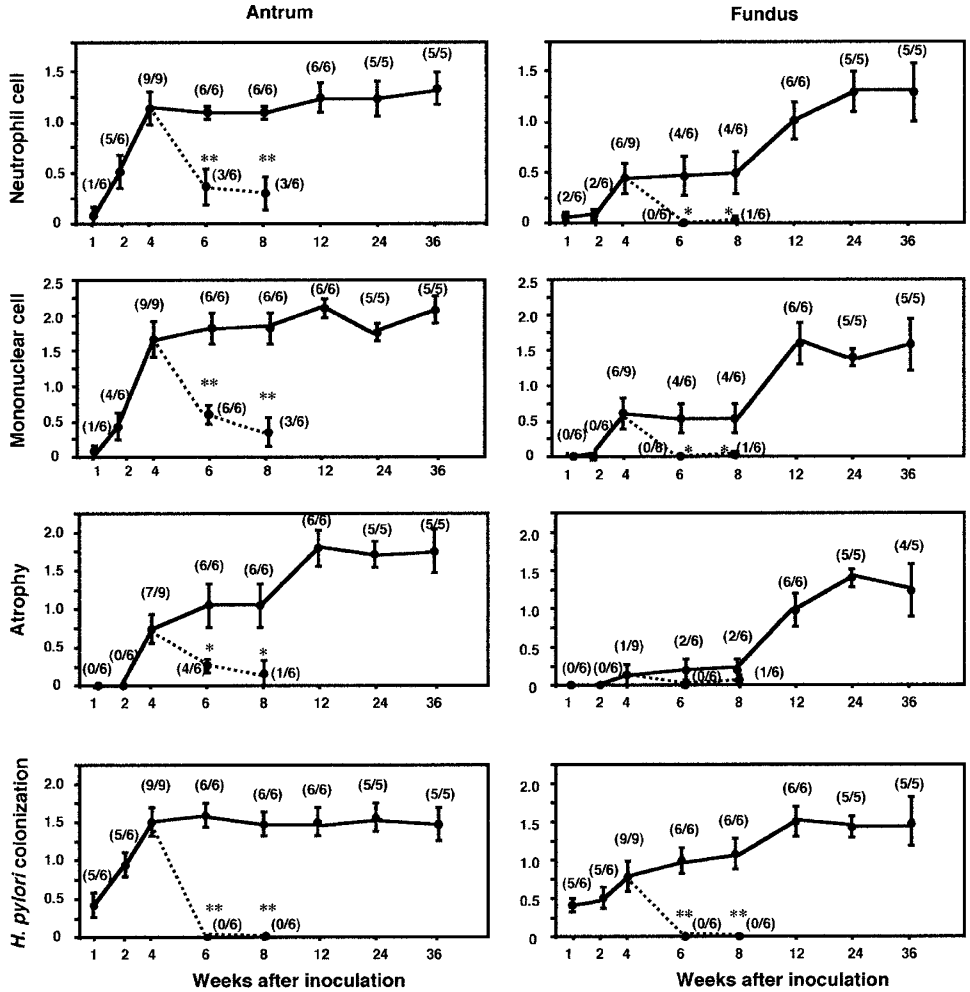

Figure 2.

Serial gastric inflammation, atrophy, and *Helicobacter pylori* colonization scores in Mongolian gerbils infected with *H. pylori* (●—●) and after *H. pylori* eradication (●···●). Histologic findings and *H. pylori* colonization in the antrum and fundus were scored from 0 to 3, as described in the "Materials and Methods" section. Values are expressed as the mean ± SEM. The numbers in parentheses are the incidences of positive findings. $*p < 0.05$, $**p < 0.01$ vs infected group at the same time point.

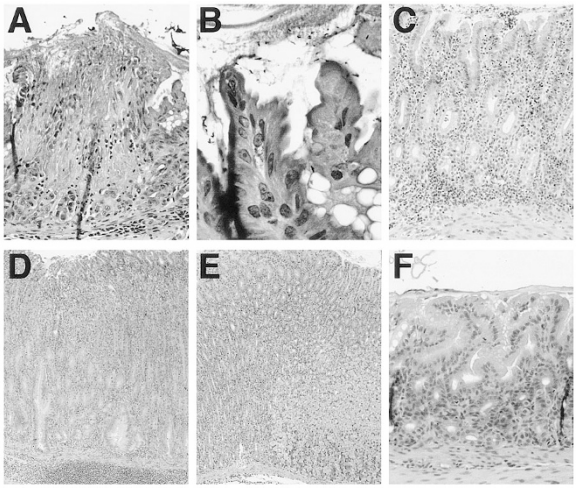

Figure 3.
Histologic findings in the stomachs of Mongolian gerbils infected with *Helicobacter pylori* and after *H. pylori* eradication. (A) Focal infiltration of neutrophils and mononuclear cells can be seen in the antral mucosa (close to the pyloric ring) 2 weeks after infection (Genta's staining, ×200). (B) At 2 weeks after infection, *H. pylori* are present in the destroyed gastric pits and the surface mucous layer (Genta's staining, ×1000). (C) Antral mucosa of gerbils infected with *H. pylori* at 4 weeks. Inflammatory cells have infiltrated the lamina propria and formed microabscesses (hematoxylin and eosin, ×200). (D) Fundic mucosa of gerbils infected with *H. pylori* at 12 weeks. Elongated glands and a submucosal lymphoid follicle can be seen (hematoxylin and eosin, ×100). (E) The border of the inflammatory and atrophic lesions in the fundic mucosa of an infected gerbil at 12 weeks. On the anal side (left side of panel), inflammatory cells have infiltrated, and the parietal cells have been destroyed. On the oral side (right side of panel), the epithelium has remained normal (hematoxylin and eosin, ×100). (F) Antral mucosa of gerbils 4 weeks after *H. pylori* eradication. In comparison with C, the inflammation is vastly improved and the epithelial structure has been recovered (hematoxylin and eosin, ×200).

mucosa of the corpus. The inflammation in the antrum peaked at 4 weeks after inoculation (Figs. 2 and 3C), whereas inflammation was only just starting to appear in the corpus at this time, reaching its peak at 12 weeks after inoculation (Figs. 2 and 3D). As shown in Figure 3E, the inflammation spread from the pyloric to the fundic mucosa. The inflammatory cell infiltration scores in both the antrum and the fundus were significantly reduced in gerbils that had undergone *H. pylori* eradication (Figs. 2 and 3F).

Atrophy

Following inflammatory cell infiltration, atrophy of the gastric mucosa was observed in the antrum of *H. pylori*–infected gerbils at 4 weeks after inoculation (Figs. 2 and 3C), whereas no remarkable atrophy was observed in the fundus at this time point. Atrophy of the fundic mucosa started to become apparent at 12 weeks after inoculation (Figs. 2 and 3D). The atrophy score in the antrum was significantly reduced in gerbils that had undergone *H. pylori* eradication (Figs. 2 and 3F).

H. pylori *Colonization*

The density of *H. pylori* colonization in the antrum and the fundus peaked at 4 and 12 weeks after inoculation, respectively. *H. pylori* colonization was not detected in the antrum nor the fundus of gerbils that had undergone *H. pylori* eradication (Fig. 2). *H. pylori* infection and its disappearance after eradication were also confirmed by culture method.

Gastric Acidity

At 1 week after inoculation, there was no difference in gastric acidity between the control and infected gerbils. However, at 2 weeks after inoculation, gastric acidity was significantly elevated in the *H. pylori*–infected group. Interestingly, gastric acidity then decreased in the *H. pylori*–infected gerbils, becoming significantly lower than in the control group at 4 weeks after inoculation and remaining significantly lower thereafter (Fig. 4). At 2 weeks after *H. pylori* eradication, gastric acidity increased almost up to the control level, and this recovery was sustained until 4 weeks after eradication (Fig. 4).

Serum Gastrin

Up to 4 weeks after inoculation, there was no significant difference in serum gastrin levels between the control and infected gerbils. However, from 6 weeks after *H. pylori* infection, the serum gastrin level started to increase significantly in the *H. pylori*–infected gerbils, reaching a peak at 24 weeks after inoculation, and remaining elevated at 36 weeks (Fig. 5). This increase in serum gastrin levels was inhibited by *H. pylori* eradication (Fig. 5).

Fundic Argyrophil Cells and Reg-Positive Cells

Very few argyrophil cells were detected in the basal regions of the fundic glands in the normal gastric mucosa (Fig. 6A), and there was no significant difference in the number of fundic argyrophil cells between the control and infected gerbils at 4 weeks after inoculation (Fig. 6C). However, the number of argyrophil cells was significantly increased in *H. pylori*–

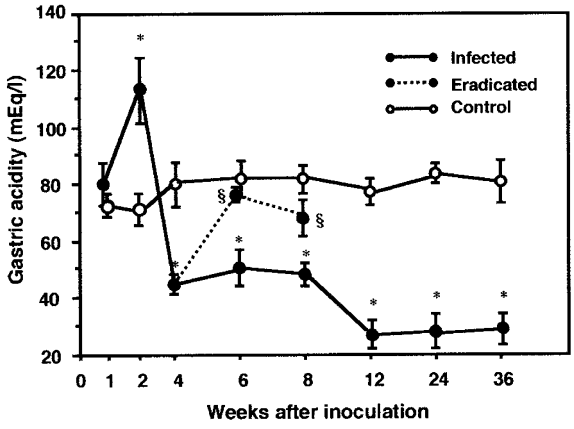

Figure 4.
Serial gastric acidity results in Mongolian gerbils infected with *Helicobacter pylori* and after *H. pylori* eradication. All results are expressed as the mean ± SEM. $^{*}p < 0.01$ vs control group at the same time point. $^{\S}p < 0.05$ vs infected group at the same time point.

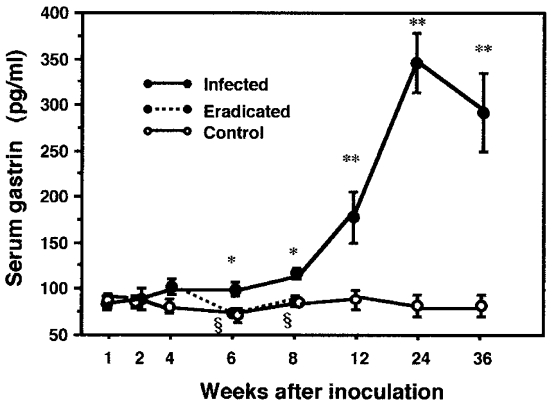

Figure 5.
Serial serum gastrin levels in Mongolian gerbils infected with *Helicobacter pylori* and after *H. pylori* eradication. All results are expressed as the mean ± SEM. $*p < 0.05$, $**p < 0.01$ vs control group at the same time point. $^{\S}p < 0.05$ vs infected group at the same time point.

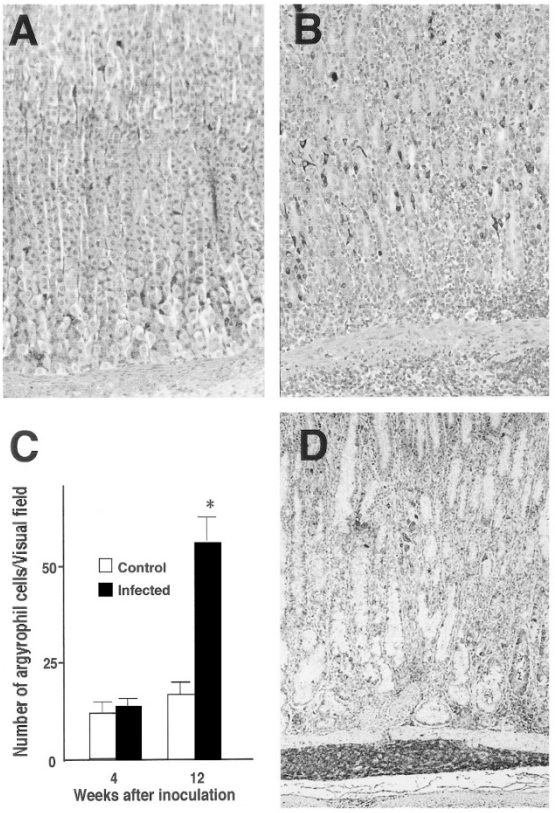

Figure 6.
Silver staining for fundic argyrophil cells. (A) Normal fundic mucosa. (B) Fundic mucosa of Mongolian gerbil infected with *Helicobacter pylori* for 12 weeks. (C) The number of fundic argyrophil cells in *H. pylori*–infected Mongolian gerbils. All results are expressed as the mean ± SEM. $*p < 0.001$ vs control group at the same time point. (D) Immunostaining for regenerating (Reg) protein in the fundus in the *H. pylori*–infected Mongolian gerbil at 36 weeks after inoculation. The number of Reg protein–positive cells was increased in the basal regions of the fundic mocosa.

infected gerbils at 12 weeks after inoculation (Fig. 6, B and C). In accordance with the increase of argyrophil cells, the number of Reg protein–positive cells was also increased in *H. pylori*–infected gerbils at more than 12 weeks after inoculation (Fig. 6D). However, Reg protein–positive cells were hardly detected in the normal fundic mucosa.

Changes in Reg *Gene Expression in the Fundus of* H. pylori*–Infected Mongolian Gerbils*

A 156-bp cDNA of Mongolian gerbil *Reg* was used as the probe for Northern blot analysis (Fig. 7). No *Reg* mRNA expression was detected in the fundus in any of the control gerbils by Northern blot analysis, although it was detected by reverse-transcription (RT)-PCR analysis (data not shown). Furthermore, no *Reg* mRNA expression was detected in the fundus in the *H. pylori*–infected gerbils within the first 2 weeks after inoculation. However, in the *H. pylori*–infected group, *Reg* mRNA became detectable in the fundus at 4 weeks after infection, and its levels continued to increase up to 36 weeks (Fig. 8). On the other hand, *H. pylori* eradication treatment diminished *Reg* mRNA expression in the fundus (Fig. 8).

Relationship between Reg mRNA Expression, Serum Gastrin Levels, and the Severity of Fundic Inflammation in H. pylori*–Infected Mongolian Gerbils*

We examined the relationship between *Reg* mRNA expression, serum gastrin levels, and the severity of fundic inflammation in *H. pylori*–infected Mongolian gerbils. Among the infected animals, serum gastrin levels correlated significantly with *Reg* mRNA expression levels in the fundus ($p < 0.001$; Fig. 9A). Fundic inflammatory cell scores for both neutrophils and mononuclear cells also correlated significantly with *Reg* mRNA expression levels in the fundus ($p < 0.005$ and 0.01, respectively) (Fig. 9, B and C).

Gastric Cell Proliferation and Apoptosis

Proliferating cell nuclear antigen (PCNA)-positive cells were observed mainly in the neck zone of the fundic

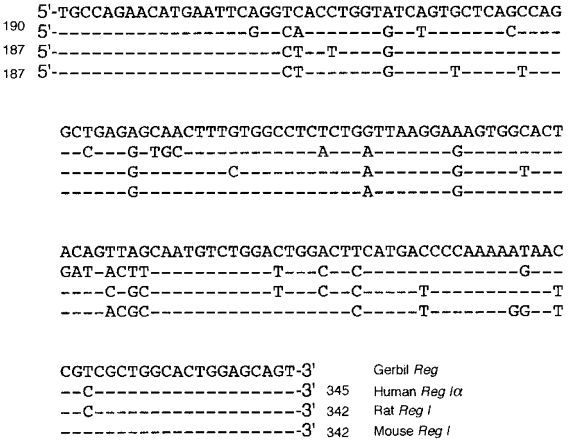

Figure 7.
Alignment of the 156-bp portion of *Reg* cDNA sequences for Mongolian gerbil. The bars in the sequences for human regenerating gene (*Reg*)*Iα*, rat *Reg I*, and mouse *Reg I* indicate oligonucleotides corresponding with the gerbil ones; oligonucleotide homologies of 83.3%, 88.5%, and 89.1% were observed between the Mongolian gerbil sequence and the human, rat, and mouse sequences, respectively.

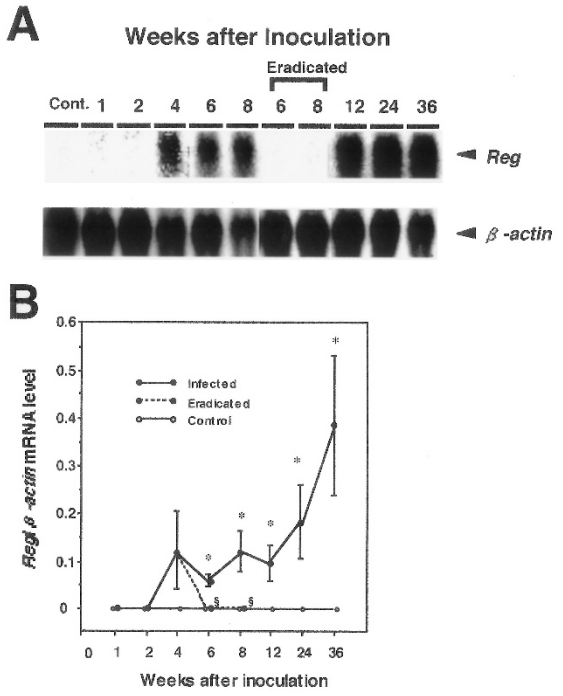

Figure 8.
Serial regenerating gene (*Reg*) mRNA expression levels in the fundus of Mongolian gerbils infected with *Helicobacter pylori* and after *H. pylori* eradication. Eradication was carried out at 4 weeks after inoculation. (A) Total fundic RNA (30 μg) was extracted at the times indicated after *H. pylori* inoculation and analyzed by Northern blotting as described in the "Materials and Methods" section. No *Reg* mRNA signal was detected among the total fundic RNA of the control gerbils at any time throughout the experiment (data not shown). (B) The ratio of the signal intensities of *Reg* to *β-actin* mRNA were quantified using a BAS 2000 image analyzer (Fujix, Tokyo, Japan). The vertical lines represent the mean ± SEM. $^{*}p < 0.05$ vs control group at the same time point. $^{\S}p < 0.05$ vs infected group at the same time point.

glands in the normal gastric mucosa. In contrast, they were distributed throughout the gastric mucosa in *H. pylori*–infected gerbils. The number of PCNA-positive cells in the fundic mucosa was significantly increased at more than 6 weeks after *H. pylori* inoculation. *H. pylori* eradication treatment reduced it to the control levels (Fig. 10).

TUNEL (terminal deoxynucleotidyl-mediated dexyuridine triphosphate-digoxigenin nick-end labeling) signal was mainly observed in the pit cells in the normal foveolar epithelium. In *H. pylori*–infected gerbils, TUNEL-positive cells were mainly distributed throughout the gastric mucosa. The number of TUNEL-positive cells in the fundic mucosa was significantly increased at more than 6 weeks after *H. pylori* inoculation. However, *H. pylori* eradication treatment reduced it to the control levels (Fig. 11).

Discussion

As we and others have previously shown, the Mongolian gerbil is a very useful animal model for studying the time courses of changes in the pathology of *H. pylori*–induced gastritis (Hirayama et al, 1996; Ikeno et al, 1999). Inflammation induced by *H. pylori* is believed to start in the pyloric mucosa and spread to the fundic mucosa. In the present study, we clearly observed this pattern of spread and, moreover, found that gastric acidity and serum gastrin levels are closely related to the pathologic changes: gastric acidity decreased in association with the spread of inflammation to the fundic mucosa, while the serum gastrin level increased in response to the reduction in gastric acidity. Regarding the reduction of gastric acidity, two possible explanations may be considered. One is that fundic inflammation may cause destruction of the acid-producing parietal cells, and the second is that ammonia generated by *H. pylori* urease may produce an alkaline environment in the stomach. In addition, recent reports suggest that IL-1β is produced in the mucosa during *H. pylori*–associated inflammation and that this may have an inhibitory effect on gastric acid secretion (Beales and Calam, 1998; Wallace et al, 1991). Thus, although we did not measure IL-1β levels in the fundic mucosa during the present study, it may be reasonable to suspect that IL-1β is also involved in the decrease in gastric acidity seen in *H. pylori*–induced gastritis.

It should be emphasized that the elevation of serum gastrin levels was preceded by the reduction in gastric acidity. Mild hypergastrinemia is generally observed in patients with chronic *H. pylori*–induced gastritis (Levi et al, 1989; Mulholland et al, 1993) and, although *H. pylori*–induced gastritis has been reported to cause hypergastrinemia either directly or indirectly via the production of various cytokines (Beales et al, 1997; Calam, 1996; Lehmann et al, 1996), our present data favor the idea that reduced gastric acidity is responsible for such hypergastrinemia.

In the uninfected control group, *Reg* mRNA was detected in the fundus by RT-PCR but not by Northern blotting, suggesting that *Reg* mRNA is expressed at very low levels in the normal fundic mucosa of Mongolian gerbils. *Reg* mRNA is normally expressed in argyrophilic ECL cells in humans and rats (Fukui et al, 1998; Higham et al, 1999). However, only a few argyrophilic cells were present in the fundus of Mongolian gerbils as assessed by silver staining, and this is compatible with the low expression of the *Reg* gene in the fundus.

Recently, we have shown that both *Reg* gene expression and the number of Reg-positive ECL cells in the rat fundic mucosa are increased during experimental hypergastrinemia evoked by proton pump inhibitors (Fukui et al, 1998). In the present study, we have clearly shown that *Reg* gene expression is augmented and both argyrophilic and Reg protein–positive cell counts are increased in the fundic mucosa during *H. pylori* infection with hypergastrinemia, suggesting a close relationship between increased *Reg* gene expression and hypergastrinemia. Interestingly, we also found that *Reg* mRNA expression levels correlated closely with the severity of fundic mucosal inflammation. As we and others have previously shown in vitro, certain cytokines including IL-6, IFN-γ, TNF-α, and cytokine-induced neutrophil chemoattractant 2β may enhance *Reg* gene transcription (Akiyama et al, 2001; Dusetti et al, 1996; Kazumori

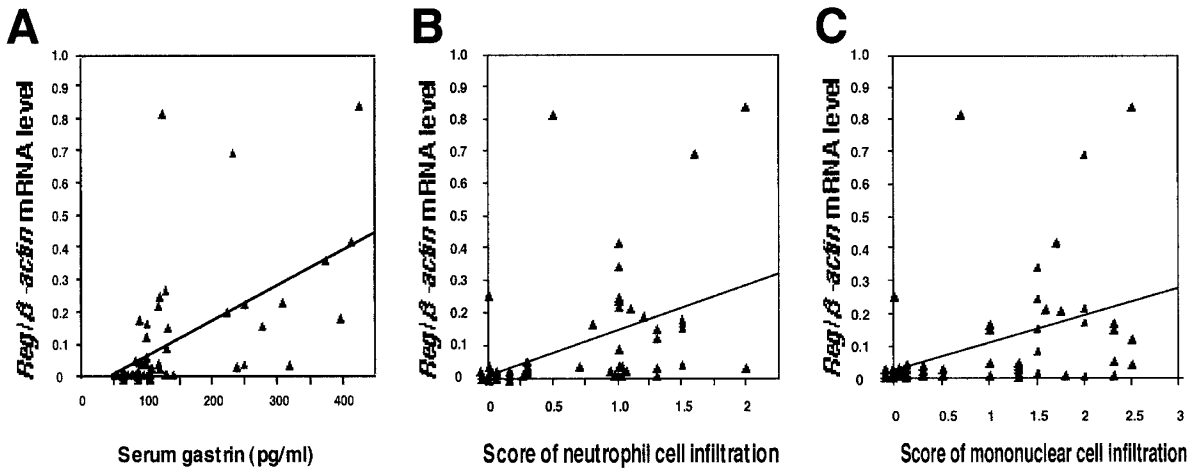

Figure 9.

Relationships between fundic regenerating gene (*Reg*) mRNA expression levels and (A) serum gastrin levels or scores for (B) neutrophil and (C) mononuclear cell infiltration in the fundus in *Helicobacter pylori*–infected Mongolian gerbils ($n = 49$). The fundic *Reg* mRNA expression levels correlated significantly with the serum gastrin level ($p < 0.001$, $r = 0.54$) and scores for neutrophil ($p < 0.005$, $r = 0.40$) and mononuclear cell ($p < 0.01$, $r = 0.37$) infiltration in the *H. pylori*–infected gerbils.

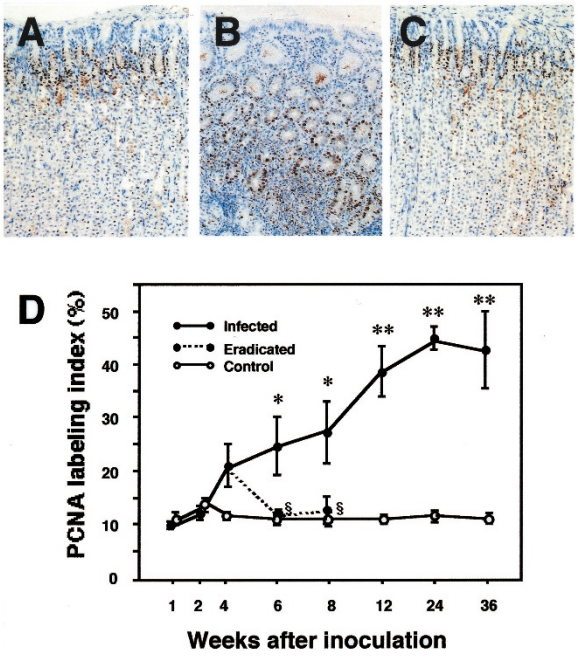

Figure 10.

Immunostaining of PCNA in the fundic mucosa of Mongolian gerbils infected with *Helicobacter pylori* and after *H. pylori* eradication. (A) Normal fundic mucosa (×100). (B) Fundic mucosa of gerbil infected with *H. pylori* for 12 weeks (×100). (C) Fundic mucosa of gerbils 4 weeks after *H. pylori* eradication (×100). (D) Serial PCNA labeling index in the fundus of Mongolian gerbils infected with *H. pylori* and after *H. pylori* eradication. All results are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs control group at the same time point. $^{\S}p < 0.05$ vs infected group at the same time point.

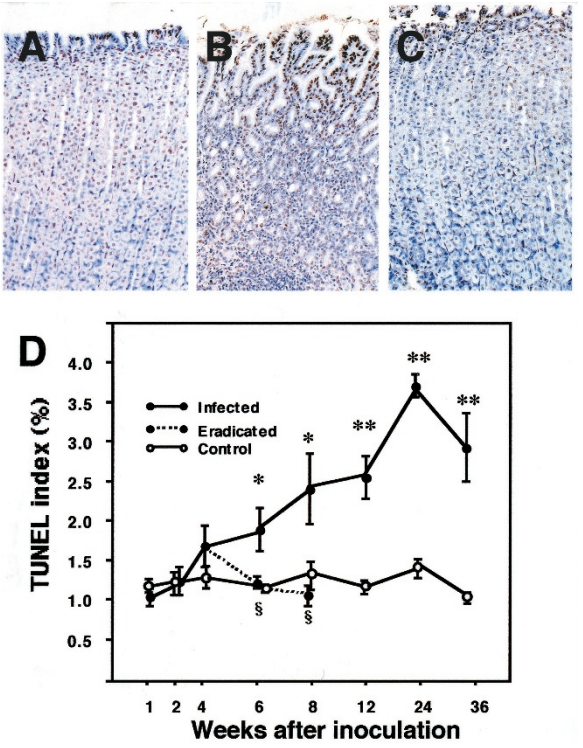

Figure 11.

TUNEL (terminal deoxynucleotidyl-mediated dexyuridine triphosphate-digoxigenin nick-end labeling) apoptotic cell detection in the fundic mucosa of Mongolian gerbils infected with *Helicobacter pylori* and after *H. pylori* eradication. (A) Normal fundic mucosa (×100). (B) Fundic mucosa of gerbils infected with *H. pylori* for 12 weeks (×100). (C) Fundic mucosa of gerbils 4 weeks after *H. pylori* eradication (×100). (D) Serial TUNEL index in the fundus of Mongolian gerbils infected with *H. pylori* and after *H. pylori* eradication. All results are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs control group at the same time point. $^{\S}p < 0.05$ vs infected group at the same time point.

et al, 2000). Because these cytokines are apparently involved in *H. pylori*–induced gastritis (Bodger and Crabtree, 1998; Calam, 1996; Genta, 1997), they (as well as hypergastrinemia) may have enhanced *Reg* gene expression in our study. On the other hand, during *H. pylori*–induced gastritis, the gastric epithelial cells are continuously being injured by inflammation and subsequently regenerated, and it has been reported that the *Reg* gene is induced during the healing of stomach tissue (Asahara et al, 1996; Kawanami et al, 1997, Kazumori et al, 2000). Thus, it is tempting to speculate that the *Reg* gene is involved in the regen-

eration of gastric epithelial cells during *H. pylori*–induced gastritis. In support of this theory, we have previously shown Reg protein to have a trophic effect on gastric epithelial cells (Fukui et al, 1998); moreover, others have demonstrated that the Reg-related protein, PAP, exerts an anti-apoptotic effect in vitro (Ortiz et al, 1998). Both proliferation and apoptosis of gastric epithelial cells are actively occurring in *H. pylori*–induced gastritis (Jones et al, 1997; Peek et al, 1997). Indeed, in our present study, we clearly showed enhancement of not only apoptosis but also proliferation of the gastric mucosal cells in *H. pylori*–infected gerbils. Thus, the *Reg* gene product may function as a trophic and/or an antiapoptotic factor in the *H. pylori*–infected gastric mucosa.

Although *H. pylori* infection is epidemiologically associated with the development of gastric cancer (Parsonnet et al, 1991), the mechanism by which this occurs is still unclear. However, because gastrin has a trophic effect on gastric epithelial cells, hypergastrinemia might play a role in *H. pylori*–induced gastric carcinogenesis. Interestingly, Wang et al (2000) have recently suggested that hypergastrinemia and *H. pylori* infection have synergistic effects that contribute toward progression to gastric cancer. Moreover, Hirayama et al (1999) have shown that poorly differentiated adenocarcinomas as well as carcinoid tumors develop during *H. pylori*–induced gastritis with hypergastrinemia. Recent studies have shown that gastrin receptors are present on gastric mucosal progenitor cells, suggesting that gastrin has direct trophic actions on gastric mucosal cells (Kazumori et al, 2001; Nakajima et al, 2002). On the other hand, several investigators have reported that gastrin promotes gastric mucosal cell proliferation indirectly by stimulating the production of growth factors such as TGF-α, heparin-binding epidermal growth factor–like growth factor, and Reg (Fukui et al, 1998; Miyazaki et al, 1999; Wang et al, 2000). Taking these possibilities together, it appears that gastrin may promote gastric tumorigenesis by acting both directly on gastric mucosal cells and indirectly by enhancing Reg protein production.

In conclusion, we have shown that *Reg* gene expression is enhanced in *H. pylori*–induced gastritis and that expression levels of this gene are closely related to serum gastrin levels and the severity of fundic mucosal inflammation. These data suggest that Reg is induced by gastrin and/or inflammation, and may promote gastric mucosal proliferation in *H. pylori*–induced gastritis.

Materials and Methods

Animals and H. pylori *Strain*

Eight-week-old male Mongolian gerbils weighing 30 to 50 g (Harlan Sprague Dawley, Inc., Indianapolis, Indiana) were used. They were housed in an air-conditioned biohazard room and had free access to food and water. The experimental protocol was approved by the Animal Care Committee of Baylor College of Medicine. *H. pylori* CA20, the strain used for inoculation of the gerbils in this study, was originally isolated from a gastric cancer patient. This strain (*cagA*$^+$, *vacA* s1m2) was identified by its morphology; Gram's staining; and its urease, catalase, and oxidase activities. Prior to inoculation, the *H. pylori* was grown in brain-heart infusion broth containing 7% fetal bovine serum at 37° C under microaerobic conditions.

Experimental Design

After fasting for 24 hours, 61 gerbils were inoculated with 1 ml culture broth containing 10^9 colony-forming units *H. pylori* via a gastric tube. A further 44 gerbils received 1 ml culture broth alone and served as uninfected controls. Four weeks after inoculation, 12 infected gerbils were administrated orally with lansoprazole, amoxicillin, and clarithromycin (10, 3, and 30 mg/kg body weight, respectively), suspended in 0.5% w/w carboxymethylcellulose sodium solution, bid for 2 days (Shimizu et al, 2000). Both infected and uninfected gerbils were killed at 1, 2, 4, 6, 8, 12, 24, and 36 weeks after inoculation. The gerbils that had undergone *H. pylori* eradication were killed at 6 and 8 weeks after inoculation (ie, 2 and 4 weeks after eradication). To ascertain *H. pylori* infection and its eradication, part of the gastric tissues were homogenized in brain-heart infusion broth and cultured as previously reported (Dore et al, 2001; Yamaoka et al, 1999). The experimental schedules, including the number of gerbils in each group killed at each time point, are summarized in Figure 1.

Gastric Acidity and Serum Gastrin Concentrations

After a 24-hour fast, the gerbils were killed and their entire gastric contents were collected by clamping the esophageal and duodenal ends of the stomach (Dial et al, 2000). The gastric juice was then separated from the gastric contents by centrifugation (10,000 rpm, 1 minute). The acidity of the gastric juice was measured by titration with 0.01 N NaOH on a titrator (TTT60 Titrator; The London Company, Ohio). In addition, blood was collected from a cardiac puncture at the time of death. The serum was stored at -20° C until assayed for gastrin as described previously (Bloom and Long, 1982).

Histopathology

After collecting the gastric contents and blood, the stomach of each gerbil was opened along the greater curvature. After macroscopic observations, the stomachs were fixed in 10% buffered formalin, sliced along the longitudinal axis into seven slips of equal width, embedded in paraffin, and cut into 4-μm sections. The sections were stained with hematoxylin and eosin for morphologic observations and by Genta's staining method for the detection of *H. pylori*, as previously described (Genta et al, 1994). The degree of gastritis in the antrum and corpus was graded according to the Updated Sydney system (Dixon et al, 1996). Histologic parameters, including neutrophil infiltration, mononuclear cell infiltration, atrophy, and density of *H. pylori*

colonization, were scored on a scale of 0 to 3 as follows: grade 0, normal (none in case of *H. pylori* colonization); grade 1, mild; grade 2, moderate; grade 3, marked. The scores were evaluated in all of the slips from each stomach, and the results were averaged. Fundic argyrophil cells were also evaluated using the standard Grimelius' silver staining method. The number of fundic argyrophil cells was counted in at least 10 well-orientated visual fields (magnification, ×100) on seven different sections, and the average per visual field was calculated. In addition, immunohistochemical staining for Reg protein was performed with the avidin-biotin-complex immunoperoxidase method using antirat Reg antibody (dilution 1:500), as previously described (Fukui et al, 1998).

Gastric Cell Proliferation and Apoptosis

The tissue sections of the fundic mucosa were used for immunostaining of PCNA. In brief, the sections were deparaffinized, rehydrated, and then pretreated with 3% H_2O_2 in methanol for 30 minutes at room temperature to quench endogenous peroxidase activity. The sections were incubated with 1% BSA in PBS (PBS) for 20 minutes and then incubated with an anti-PCNA antibody (PC10, Dako, Kyoto, Japan; dilution, 1:500) for 60 minutes at 37° C. Thereafter, sections were immunostained using a LSAB kit (Dako, Marseille, France) in accordance with the supplied protocol, visualized by 3,3'-diaminobenzidine tetrahydrochloride, and counterstained with Mayer hematoxylin. Five-hundred epithelial cells were counted in five different visual fields (magnification, ×100) on each section. PCNA labeling index was calculated as the percentage of positive cells.

The tissue sections of the fundic mucosa were also used for TUNEL assay using an in situ cell death detection kit (Roche, Mannheim, Germany). In brief, the sections were deparaffinized, rehydrated, and then incubated with proteinase K (20 μg/ml) for 15 minutes at 37° C. The sections were then washed in PBS, and endogenous peroxidase was blocked by 3% H_2O_2 in methanol for 30 minutes at room temperature. After washing in PBS, slides were incubated with terminal transferase buffer and reaction mixture for 60 minutes at 37° C. The sections were then washed in PBS and incubated with peroxidase-conjugated Fab fragments of anti-fluorescein at 37° C for 30 minutes. The slides were washed in PBS, visualized by 3,3'-diaminobenzidine tetrahydrochloride, and counterstained with Mayer hematoxylin. Five-hundred epithelial cells were counted in five different visual fields (magnification, ×100) on each section. TUNEL index was calculated as the percentage of positive cells.

Sequence Analysis of Mongolian Gerbil Reg Gene

Total RNA was isolated from the pancreas of a Mongolian gerbil using Trizol Reagent (Gibco BRL, Rockville, Maryland). To generate cDNA, 4 μg of total RNA was reverse-transcribed using 200 U of SuperScript II RT (Gibco BRL) in a total reaction volume of 20 μL. Then, to identify Mongolian gerbil *Reg* cDNA clones, we searched similar parts of the sequence among murine, rat, and human *Reg* cDNA and chose a forward primer (corresponding to human *Reg* positions 190–206) and a reverse primer (positions 328–345) for PCR (Fig. 7). One μl of reverse-transcription product (cDNA) was amplified by PCR in a 20-μl reaction volume containing 40 pmol of above set of primers, 1.25 U of Ampli-*Taq*DNA polymerase (Applied Biosystems, Brunchburg, New Jersey), and final concentration of PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 2.5 mM $MgCl_2$, 10 mM DTT, and 1 mM dNTP. The PCR amplification was performed as follows: at 95° C for 5 minutes, once; 35 cycles of 94° C for 30 seconds, 50° C for 30 seconds, and 72° C for 1 minute; and then at 72° C for 7 minutes. The PCR product was ligated into pGEM-T Easy vector (Promega, Madison, Wisconsin), and the nucreotide sequence of the insert was determined by the dideoxy chain termination procedure (Sanger et al, 1977) (Fig. 7).

Northern Blot Analysis

Extracted RNA from the fundus of Mongolian gerbils was separated by electrophoresis in 0.66 M formaldehyde/1% agarose gel. After transfer to a nitrocellulose membrane (Schleicher & Schuell, Germany), the nucleic acids were fixed to the membrane by UV crosslinking. The probes used for Northern blot analysis were a 156-bp cDNA of Mongolian gerbil *Reg* (Fig. 7) and a 164-bp cDNA of Mongolian gerbil *β-actin* (Takashima et al, 2001). Radiolabeling of the probes was performed with [α-^{32}P]deoxycytidine triphosphate using a random primer labeling kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Hybridization was performed at 42° C, and filters were washed twice at 55° C in 0.1 × standard saline citrate plus 0.1% sodium dodecyl sulfate as described previously (Fukui et al, 1998). The radiolabeled DNA probe was detected by a bioimage analyzer (BAS 2000, Fujix, Tokyo, Japan).

Statistical Analysis

All values were expressed as the mean ± SEM. Statistical differences between two animal groups were assessed by the unpaired Student's *t* test or Mann-Whitney *U* test when data was not parametric. The relationship between *Reg* mRNA levels and serum gastrin levels or fundic inflammatory scores were assessed by linear regression analysis.

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