Helicobacter pylori infection potentiates aspirin induced gastric mucosal injury in Mongolian gerbils

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Background: Helicobacter pylori infection and non-steroidal anti-inflammatory drugs are two major causes of gastric ulceration but interactions between H pylori and these drugs in gastric mucosal injury are unclear.

Aims: We studied the influence of experimental H pylori infection on gastric mucosal injury induced by aspirin.

Subjects: Male Mongolian gerbils free of specific pathogens were used.

Methods: H pylori ATCC43504 culture broth was administered by oral gavage at seven weeks of age. After three weeks, acidified aspirin (400 mg/kg) was administered orally, and three hours later the total area of gastric erosions, myeloperoxidase (MPO) activity (an index of neutrophil accumulation), thiobarbituric acid reactive substances (TBARS, an index of lipid peroxidation), and KC/GRO (a chemoattractive cytokine in rodents) were measured in gastric mucosa. To determine the role of neutrophils in these circumstances, antigerbil neutrophil rabbit serum (ANS) was administered to some animals 18 hours before aspirin.

Results: Aspirin caused more extensive hemorrhagic erosions (33.1 (12.3) mm²) associated with greater MPO activity (1887.7 (598.5) μU/mg protein) and TBARS (0.33 (0.14) nmol/mg protein) and KC/GRO concentrations (28.3 (9.5) pg/mg protein) in infected than in uninfected gerbils (13.7 (2.3); 204.0 (68.9); 0.12 (0.06); 3.1 (0.8), respectively). Pretreatment with ANS inhibited the increases in gastric erosions, MPO activity, and TBARS but not KC/GRO concentration. The reduction in aspirin induced mucosal injury by administration of ANS was much greater in H pylori infected animals (65%) than in uninfected animals (31%).

Conclusions: H pylori infection potentiates aspirin induced gastric mucosal injury by mechanisms that include accumulation of activated neutrophils.

Both Helicobacter pylori infection and non-steroidal anti-inflammatory drug (NSAID) use are well established risk factors for gastrointestinal mucosal injury. Several clinical studies have explored the relationships between these two factors. Very few experimental data have been reported however, leaving many questions unanswered.

Among the clinical studies, Santucci and colleagues found that gastroduodenal mucosal lesions caused by four weeks of NSAID administration were more severe if patients had H pylori infection. In addition, Publig and colleagues reported that the prevalence of H pylori infection in patients taking NSAIDs who developed gastric ulcers was 83%, significantly greater than in patients without gastric ulcers (45%). However, other studies found no evidence of potentiated injury when H pylori infection coexisted with NSAID use. Among patients with rheumatoid arthritis taking NSAIDs, Graham and colleagues reported gastric erosions in 34% and bleeding in 32% of patients who also had H pylori infection, representing lower incidences than in patients without H pylori infection (57% and 61%, respectively). Lipscomb and colleagues found no difference in the prevalence of H pylori infection on severity of gastric mucosal injury in healthy volunteers who received NSAIDs for up to four weeks. These disagreements may be related to differences in severity of gastric mucosal atrophy between patients with and without H pylori infection as well as differences in the basal level of mucosal inflammation.

Recent experimental studies have indicated that neutrophils adhere to the endothelium via various adhesion molecules are involved in the development of gastric mucosal injury induced by H pylori infection or NSAID use. We previously reported that H pylori and NSAIDs cause neutrophils to express adhesion molecules followed by accumulation of neutrophils into the gastric mucosa. Activated neutrophils have been suggested to injure endothelial and epithelial cells by producing active oxygen species and proteases. Additionally, in a randomised controlled trial, Taha et al demonstrated that gastric neutrophils associated with H pylori infection increased the incidence of ulceration in long term NSAID users. These findings suggest that H pylori and NSAIDs can elicit an acute inflammatory response in the gastric mucosa leading to neutrophil mediated tissue injury.

The objective of the present study was to determine the influences on and consequences of neutrophil associated inflammatory reactions in gastric mucosa exposed to both experimental H pylori infection and NSAIDs. In 1993, Hirayama and colleagues described a model in which Mongolian gerbils infected with H pylori developed pathological changes in the stomach that mimicked those seen in humans who harbour the bacteria. These changes included a high incidence of gastritis after six weeks of infection and gastric ulceration after six months of infection. In addition, Watanabe and colleagues found that inoculation of H pylori isolated from a patient with a gastric ulcer could result in the occurrence of gastric cancer in Mongolian gerbils. Hence the gerbil model can be used to clarify the pathophysiology of H pylori induced gastric mucosal lesions. We chose these animals for our present experiments.

Abbreviations: MPO, myeloperoxidase; TBARS, thiobarbituric acid reactive substances; ANS, antineutrophil serum; NSAID, non-steroidal anti-inflammatory drug; CMC, carboxymethylcellulose; IL, interleukin.
Aspirin induced gastritis and *H. pylori*

**MATERIALS AND METHODS**

**Animals and bacteria**

Seven week old male Mongolian gerbils (MGs/Sea) free of specific pathogens were purchased from Seiwa Experimental Animals (Fukuoka, Japan). *H. pylori* (ATCC43504) was obtained from the American Type Culture Collection (Rockville, Maryland, USA). All experimental procedures were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine.

**Bacterial inoculation**

*H. pylori* was grown in 200 ml Erlenmeyer flasks containing Brucella broth (IBL, Cockeysville, Maryland, USA) supplemented with 10% heat inactivated horse serum (Nakarai Tesque, Kyoto, Japan). The flasks were incubated at 37°C for 24 hours in an 8% CO₂ atmosphere on a rotary shaker. Broth culture (500 µl) containing *H. pylori* (4x10⁶ CFU/ml) was given to gerbils by oral gavage after a 24 hour fast.

**Aspirin induced gastric mucosal injury**

Thirty six gerbils were divided into four groups of nine animals. Three weeks after inoculation with *H. pylori*, aspirin (400 mg/kg; Sigma Chemical, St Louis, Missouri, USA) suspended in 0.8 ml of 0.25% carboxymethylcellulose (CMC) and 0.1 N HCl were administered orally to gerbils fasted for 18 hours. In the control group, gerbils received 0.8 ml CMC alone. At three hours after aspirin administration, gerbils were killed by exsanguination during urethane anaesthesia. The stomach was removed and opened along the greater curvature. Macroscopic gastric damage was examined under a dissecting microscope using a square grid, and was quantified as the total area of haemorrhagic erosions (erosion index).

A small piece of gastric mucosa from each animal was plated on Skirrow agar containing 7% horse blood. After incubation in an 8% CO₂ atmosphere, the organisms were identified as *H. pylori* based on colony morphology, Gram stain, and production of urease, oxidase, and catalase. In three gerbils from each group, the remainder of the mucosa was fixed in 10% neutral buffered formalin for histopathological examination. In the other six gerbils from each group, the remainder of the mucosa was scraped off with two glass slides and homogenised in 1.5 ml of 10 mM potassium phosphate buffer (pH 7.8) using a Teflon Potter-Elvehjem homogeniser. The homogenate was used to measure the concentration of thiobarbituric acid reactive substances (TBARS) and also malondialdehyde (MDA) activity in the gastric mucosa, an index of neutrophil infiltration.

**Detection of *H. pylori* infection**

At three weeks, *H. pylori* was cultured and identified on the basis of colony morphology, Gram stain, and production of urease, oxidase, and catalase in all gerbils inoculated with the organism.

**RESULTS**

**Detection of *H. pylori* infection**

Mild oedema and erythema were observed macroscopically in the gastric mucosa of gerbils inoculated with *H. pylori*. Oral administration of aspirin produced mild haemorrhagic erosions except in *H. pylori* infected gerbils where severe haemorrhagic erosions resulted.

**Erosion index**

Figure 1 shows the index of gastric erosion for gerbils exposed to *H. pylori* and/or aspirin. The erosion index in gerbils receiving aspirin was significantly greater than that in untreated control gerbils. In addition, oral administration of aspirin to *H. pylori* infected gerbils resulted in a mean erosion index 2.5 times greater than in aspirin treated gerbils without *H. pylori* infection.

**Microscopic findings**

Microscopic examination revealed neutrophils and mononuclear cells in the gastric lamina propria in gerbils infected with *H. pylori*. In gerbils who received only aspirin, occasional neutrophils and mild erosions were observed. In *H. pylori* infected gerbils, administration of aspirin resulted in intense infiltration by neutrophils and severe erosions of the gastric mucosa (fig 2).

![Figure 1](http://gut.bmj.com/)

**Figure 1** Erosion index for the gastric mucosa of *Helicobacter pylori* (HP) and/or aspirin (Asp) treated gerbils. Three weeks after inoculation of HP, gerbils were given Asp. Three hours later, the stomach was removed and gastric damage was quantified as the total area of haemorrhagic erosions (erosion index). Control gerbils received only carboxymethylcellulose without HP. Values are mean (SEM) for six gerbils. *p<0.05 compared with gerbils who received Asp alone.
MPO activity in the gastric mucosa

As shown in fig 3, MPO activity in the gastric mucosa of H. pylori infected gerbils was significantly greater than in controls. However, mucosal MPO activity in gerbils treated with aspirin alone was unchanged. MPO activity in gerbils exposed to both H. pylori and aspirin was significantly greater than in gerbils who received either H. pylori or aspirin alone.

KC content in the gastric mucosa

As shown in fig 4, KC content in the gastric mucosa of gerbils inoculated with H. pylori was significantly greater than that in controls. Administration of aspirin alone to gerbils had no significant effect on KC content in the gastric mucosa. Mucosal KC content in gerbils receiving both H. pylori and aspirin was significantly greater than that in gerbils receiving either H. pylori or aspirin alone.

TBARS content in the gastric mucosa

The TBARS content in the gastric mucosa of gerbils treated with either H. pylori or aspirin alone was not increased relative to TBARS in untreated control gerbils. However, TBARS content in the gastric mucosa of gerbils treated with both H. pylori and aspirin was significantly greater than in gerbils treated with either H. pylori or aspirin alone (fig 5).

Effect of ANS

The circulating neutrophil count showed a decrease of 77.2%, 18 hours after administration of ANS (1660 (260) v 7300 (840)/mm³ in gerbils treated with normal rabbit serum; p<0.01). Platelet count was not influenced by ANS administration. Erosion index, MPO activity, and TBARS were significantly less in neutrophil depleted gerbils exposed to H. pylori plus aspirin than in similarly exposed animals treated with normal rabbit serum (figs 6, 7). However, administration of ANS had no influence on the increase in KC content. In contrast, aspirin induced mucosal injury in uninfected animals was also reduced after administration of ANS. The inhibitory effect on mucosal damage by ANS administration was much

Figure 2 Microscopic features of the pyloric mucosa in gerbils. (A) Helicobacter pylori group. Moderate amounts of neutrophils and mononuclear cells were seen three weeks after inoculation of H. pylori. (B) H. pylori and aspirin group. Numerous neutrophils and mononuclear cells as well as deep erosions were evident (haematoxylin and eosin stain, ×100).

Figure 3 Myeloperoxidase (MPO) activity in gastric mucosa in Helicobacter pylori (HP) and/or aspirin (Asp) exposed gerbils. HP and/or Asp induced gastric mucosal injury was produced as described for fig 1. MPO activity in the gastric mucosa was determined by a modification of the method of Krawisz and colleagues. Values are mean (SEM) for six gerbils. *p<0.05 compared with control gerbils with neither HP infection nor Asp administration; ††p<0.01 compared with gerbils with HP infection or treatment with Asp alone.

Figure 4 KC concentration in gastric mucosa in Helicobacter pylori (HP) and/or aspirin (Asp) exposed gerbils. HP and/or Asp related gastric mucosal injury was induced as described for fig 1. KC in gastric mucosa was measured by an enzyme immunoassay. Values are mean (SEM) for six gerbils. *p<0.05 compared with control gerbils with neither HP infection nor Asp administration; ††p<0.01 compared with gerbils with HP infection or treatment with Asp alone.
Aspirin on the gastric mucosa may be potentiated by uninfected animals. These findings indicate that the effects of aspirin and/or *Helicobacter pylori* chemoattractant in aspirin and/or aspirin (Asp) exposed gerbils. HP and/or Asp induced gastric mucosal injury was produced as described for fig 1. TBARS in the gastric mucosa were measured by the method of Ohkawa and colleagues. Values are mean (SEM) for six gerbils. *p<0.05 compared with gerbils with only HP infection or Asp treatment.

**DISCUSSION**

In the present study, haemorrhagic erosions in the gastric mucosa caused by aspirin were much more severe in gerbils with *H pylori* infection than in uninfected gerbils. Recently, neutrophils have been implicated in the pathogenesis of NSAID induced gastric mucosal injury. In the present study we therefore evaluated neutrophil infiltration and neutrophil chemoattractant in aspirin and/or *H pylori* induced gastric mucosal injury. While minimal neutrophil infiltration resulted when only aspirin was administered, neutrophil infiltration was more prominent when *H pylori* infection was also present. In addition, aggravation of gastric mucosal lesions induced by the combination of aspirin and *H pylori* infection was significantly inhibited in neutrophil depleted gerbils. The reduction in mucosal injury by administration of ANS was much greater in *H pylori* infected animals than in uninfected animals. These findings indicate that the effects of aspirin on the gastric mucosa may be potentiated by *H pylori* infection via neutrophil dependent mechanisms. Our previous in vitro studies showed that aspirin caused adherence of neutrophils to endothelial cells by increasing surface expression of CD11b/CD18 on neutrophils; adherence was followed by neutrophil mediated endothelial cell injury. Asako and colleagues reported that superfusion of aspirin or indomethacin promoted neutrophil adhesion to postcapillary venules of rat mesenteric vessels but did not result in trans-endothelial migration of neutrophils. In addition, Wallace and colleagues as well as our own investigational group have demonstrated that NSAID induced gastric mucosal injury in rats can be prevented by inhibiting neutrophil-endothelial cell adhesion using monoclonal antibodies directed against adhesion molecules. Our recent experiments implicated neutrophils adhering to blood vessels, but not neutrophils migrating into the interstitium, in uncomplicated aspirin induced gastric mucosal injury. Thus neutrophil-endothelial cell adhesion appears to be an early event in the pathogenesis of NSAID induced gastric mucosal injury.

In contrast, *H pylori* associated gastric mucosal injury has been attributed to activated neutrophils that adhere to postcapillary venules and subsequently migrate into the interstitium. Accumulation of neutrophils in *H pylori* infected human gastric mucosa is related to increased concentrations of interleukin (IL)-8, a potent neutrophil chemoattractant that is released by gastric epithelial cells. In the present study, we measured KC, an IL-8-like neutrophil chemoattractant in gastric mucosa. KC is considered to be both a potent chemoattractant and an upregulator of CD11b/CD18 cell surface expression in rodent neutrophils. Bozic and colleagues reported that KC mRNA is constitutively expressed in various tissues. We found that gastric mucosal KC content in gerbils exposed to *H pylori* alone was somewhat greater than in mucosa exposed to aspirin alone. Furthermore, in gerbils treated with aspirin after inoculation with *H pylori*, the KC content in gastric mucosa was significantly greater than in gastric mucosa of gerbils that received only aspirin. Greater than in gastric mucosa of gerbils that received only aspirin. Furthermore, in gerbils treated with aspirin after inoculation with *H pylori*, the KC content in gastric mucosa was significantly greater than in gastric mucosa of gerbils that received only aspirin. These findings suggest that increased KC content may be involved in accumulation of neutrophils in the gastric mucosa. In the present study, administration of antiserum against neutrophils remarkably reduced the increase in mucosal MPO activity but not the increase in KC content. Neutrophils therefore were not the main source of KC. While the source of KC was not identified in this study, KC may have been produced by gastric epithelial cells and/or macrophages. In addition, the
mechanism by which KC content was particularly increased in gerbil gastric mucosa exposed to both aspirin and H pylori resulted in increased neutrophil adherence to endothelial cells. Further studies are needed to inves-
tigate regulation of KC in the gerbil gastric mucosa. Overall, we hypothesised that administration of aspirin to H pylori infected mucosa caused adherent neutrophils to easily migrate into the extravascular space by chemotractants such as KC.

Oxygen derived free radicals are known to cause peroxidation of polyunsaturated fatty acids in cell membranes. The resulting increase in lipid peroxides can lead to changes in membrane fluidity and permeability, and finally to cell lysis. We previously reported that lipid peroxidation plays an important role in the pathogenesis of gastric mucosal lesions induced by burn stress, platelet activation factor, and ischaemia-reperfusion or NSAIDs. In the present study, TBARS in gastric mucosa, an index of lipid peroxidation, were more abundant in gels with both H pylori infection and aspirin administration than in gels treated with either H pylori or aspirin alone. These findings indicate that lipid peroxidation, most likely caused by neutrophil derived oxygen free radicals, was easily induced in the gastric mucosa of ger-

As a summary, the present study indicated that administra-
tion of aspirin to gels for three weeks after H pylori inoculation produced severe gastric mucosal injury via marked infiltration of neutrophils. Hence eradication of H pylori could help prevent NSAID induced mucosal injury, as also indicated by a recent clinical trial where eradication of H pylori before NSAID use reduced the occurrence of NSAID induced ulcers. In the present study, three weeks after H pylori inoculation, gels exhibited typical gastritis with neutrophil and mononuclear cell infiltration in the lamina propria and a few superficial erosions. The Mongolian gerbil model subsequently showed characteristic changes of chronic gastritis, including forma-
tion of lymphoid follicles six weeks after H pylori infection and gastric ulcers at more than six months after infection. The effect of NSAID administration on chronic gastritis or ulcers, showing such mononuclear cell mediated chronic inflamma-
tion, is of considerable importance. The effects of an NSAID in gerbils at six weeks and six months following inoculation of H pylori need to be investigated.

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