MODEL OF INFECTION

Induction of ulceration and severe gastritis in Mongolian gerbil by *Helicobacter pylori* infection

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Specific pathogen-free Mongolian gerbils were infected orally with *Helicobacter pylori* to establish a new small animal model of severe gastritis. *H. pylori* was recovered by culture from both antrum and body over a 16-week period after a single inoculation. The number of *H. pylori* colonising the antrum was about 100-fold higher than in the body, and this was consistent throughout the experiment. Histological examination showed that all animals developed severe inflammation with infiltration of polymorphonuclear leucocytes and mononuclear cells into the lamina propria and submucosa of the antrum from 4 weeks after infection. From 8 weeks after infection, multifocal lymphoid follicles appeared in the lamina propria and submucosa, and micro-erosions were also observed in the epithelial layer. At 16 weeks after infection, ulceration with disruption of the lamina muscularis mucosae was observed in the antral mucosa. To determine whether *H. pylori* caused gastritis or not, infected gerbils were treated with amoxycillin. After the treatment, gastritis could not be seen in the gastric mucosa. Therefore, the Mongolian gerbil is a useful small animal model to study the pathogenesis of *H. pylori* in gastric ulceration and severe gastritis and to assess anti-*H. pylori* treatment.

Introduction

Warren and Marshall [1] first isolated *Helicobacter pylori* in 1983 from the gastric mucosa of patients with chronic active gastritis and since then the association between this micro-organism and gastric diseases has been widely studied [2–4]. In man, inoculation of *H. pylori* results in gastritis with severe infiltration of polymorphonuclear leucocytes and mononuclear cells into the gastric mucosa but not erosion and ulcer formation [5, 6]. Therefore, it is clear that *H. pylori* is a causative agent of active gastritis in man. From more recent data, *H. pylori* is considered to be implicated in the development of peptic ulcer and gastric carcinoma as well as gastritis.

There are several animal models available to help understand the pathogenesis of *H. pylori* infection, including gnotobiotic piglets, athymic mice, germ-free euthymic mice and monkeys [7–10]. However, these models are not easily constructed, handled and main-

tained in ordinary laboratories. The mouse model is possibly more useful than the other models. However, *H. pylori* strains isolated from human gastric mucosa produced only mild histological changes in the mouse mucosa. Therefore, adaptation to animals was attempted to cause active gastritis [11], which was still insufficient as compared with *H. pylori*-induced inflammation in man and that in mice or ferrets induced by other *Helicobacter* species such as *H. felis*, *H. mustelae* or *Gastrospirillum hominis* (*H. heilmannii*) originating from cat, ferret or cheetah, respectively [12–14]. These *H. pylori*-like organisms are somewhat different from *H. pylori* morphologically, genetically and biochemically [15].

A Mongolian gerbil model was developed to establish a small animal model for severe inflammation and obvious ulceration by *H. pylori* and to evaluate anti-*H. pylori* therapy. Current data indicated that severe inflammation and ulceration could be observed in the antral mucosa of Mongolian gerbils. The present study attempted to eradicate *H. pylori* and examine whether amoxycillin had any effect on gastritis, in order to verify that the inflammation was induced by *H. pylori* in this model.

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Materials and methods

Test animals

Four-week-old specific pathogen-free male Mongolian gerbils (MGS/sea, 45–55 g; Seiwa Experimental Animal; Koiwai, Yositori-cho, Chikuzo-Gun, Fukuoka, Japan) were used in this study; they were free of infection with *Bordetella bronchiseptica*, *Corynebacterium kutschri*, *Salmonella* spp., *Mycoplasma pulmonis*, *Streptococcus pneumoniae*, *Pasteurella pneumotropica*, *Pseudomonas aeruginosa*, *Giardia* spp., *Spironucleus* spp., *Syphacia* spp., mouse hepatitis virus, Sendai virus and Tyzzer's organism as evidenced by culture, microscopic and serological testing by the breeder. The gerbils were housed in animal facilities and given food and water *ad libitum*. The study was approved by the Animal Experiment Committee for Fujisawa Pharmaceutical Co., Ltd.

Bacterial strains and growth conditions

H. pylori strains CPY2052, HPK127 and HPK6 were isolated from gastric biopsy samples from patients with duodenal ulcers and with severe inflammation indicated by another biopsy specimen in the same patients. These strains were identified by morphology, Gram's stain, urease, oxidase and catalase production; resistance to nalidixic acid and sensitivity to cephalothin, and as *cagA*⁺ (cytotoxin-associated gene A) and *tox*⁺ (vacuolating cytotoxin) strains by colony hybridisation [16] with PCR amplified *cagA*-specific radiolabeled probe [17] and cytotoxin assay [18]. Bacteria were grown in Brucella Broth (Becton Dickinson, Cockeysville, MD, USA) containing fetal bovine serum 10% v/v for 24–30 h at 37°C under CO₂ 10%, with shaking (120 rpm) and stored at –70°C in Brucella broth containing glycerol 15%.

Experimental design

Two ml of a 1×10^8 cfu/ml suspension of *H. pylori* HPK127 in Brucella broth were inoculated orally into each of 23 Mongolian gerbils which had been fasted overnight. A further 19 gerbils were inoculated with Brucella broth as controls. Four or five of the infected gerbils and three or four of the non-infected control gerbils were killed with CO₂ at 1, 2, 4, 8 and 16 weeks after infection. The stomach of each animal was removed and opened for macroscopic observation. For half of each gastric mucosa, the antrum and the body were scraped separately and homogenised in 1 ml of 0.1 M phosphate-buffered saline. The remainder of the stomach samples were used for histological examination. Samples (0.1 ml) of homogenate were inoculated on to Brucella agar plates containing horse serum 3% v/v, starch 2% w/v, Skirrow's antibiotics supplement (vancomycin 10 µg/ml, polymyxin B 2.5 IU/ml and trimethoprim 5 µg/ml; Unipath Ltd, Basingstoke), nalidixic acid (Nacalai Tesque Inc., Osaka, Japan) 10 µg/ml and bacitracin (Sigma) 30 µg/ml. All plates

were incubated at 37°C under CO₂ 10% for 5 days. All colonies on the plates were examined for urease activity by phenol red indicator paper which was soaked in 0.01 M phosphate buffer containing urea 10% w/v and phenol red 0.01% w/v. Colonies picked up from the plate were also identified by the organism's typical morphology. *H. pylori* CPY2052 or HPK6 were also inoculated into four gerbils, respectively, which were killed at 4 weeks after infection as described above.

Treatment of infected gerbils

Twelve gerbils infected with *H. pylori* HPK127 as described above were used in this study. At 4 weeks after infection, the gerbils were divided into three groups of four. Each of two groups were treated orally with 3.2 or 0.32 mg of amoxicillin/kg (Fujisawa Pharmaceutical Co. Ltd, Osaka, Japan), suspended in methyl cellulose solution 0.5% w/v for 10 days, three times a day. One group was treated with the vehicle only. One day after the final treatment, the gerbils were killed and the number of *H. pylori* in the antrum and the body was counted as described above.

Histological examination

Half of the gastric mucosa was immersed in buffered formalin 10%. The specimens were processed by standard methods, embedded in paraffin, sectioned and stained with haematoxylin and eosin and Giemsa stain [7].

Results

Colonisation efficiency

To explain the distribution of *H. pylori* in different parts of the gastric mucosa, the number of organisms was counted both in the antrum and the body. All colonies grown on the plates were *H. pylori*, identified according to urease activity by phenol red indicator paper and spiral morphology (data not shown). *H. pylori* HPK127 colonised both the antrum and the body of Mongolian gerbils for 16 weeks (Fig. 1). For 1–2 weeks, the number of *H. pylori* in the antral mucosa increased rapidly, from 3×10^3 to 3×10^5 cfu/tissue sample, and then the number of colonies increased 10-fold (3×10^6 cfu/tissue sample) by 16 weeks after infection. The number of *H. pylori* colonising the body was *c.* 100-fold less than that in the antrum. Two other clinical isolates (*H. pylori* CPY2052 and HPK6) also colonised the gastric mucosa of gerbils in similar numbers with gastritis at 4 weeks after infection (data not shown). Neither *H. pylori* nor other urease-positive organisms were cultured from the gastric mucosa of non-infected gerbils (data not shown), and the dominant micro-organisms in the gastric mucosa were *Lactobacillus* spp. (unpublished observations).

Fig. 1. The number of *H. pylori* in the antral (●) and body mucosa (○) of Mongolian gerbils infected with *H. pylori* HPK127. Each bar indicates SD of the mean.

Macroscopic findings

At 1 and 2 weeks after infection, no remarkable change was shown in the gastric mucosa of any gerbils infected with *H. pylori* HPK127 and non-infected control gerbils. In contrast, at 4 weeks after infection, swelling of the antral mucosa close to the duodenum was observed in all infected gerbils. This change in the antral mucosa increased from 4 weeks to 16 weeks after infection (data not shown). These results were consistent with the distribution and growth curve of *H. pylori* in the gastric mucosa of the gerbils. However, it was difficult to observe ulceration (as described below) macroscopically because of the large amounts of mucus on the gastric mucosa at 16 weeks after infection.

Histopathological findings

A summary of the histopathological changes in the gastric mucosa of Mongolian gerbils infected with *H.*

pylori HPK127 is shown in Table 1. Throughout this experiment, minimum infiltration of inflammatory cells was observed in the gastric mucosa of control gerbils (Fig. 2A) and no *H. pylori*-like organisms were seen in the specimens from non-infected gerbils (data not shown). At 1 week after infection, the gastric mucosa of all gerbils was similar to that of the control group. At 2 weeks after infection, infiltrations of polymorphonuclear leucocytes and mononuclear cells were observed in the lamina propria of the antrum of all gerbils, mainly close to the duodenum but also in the submucosa of the antrum of two of the gerbils tested (Fig. 2B), although there was no inflammation in the body mucosa. However, the inflammation in the antrum did not damage the covering epithelial cells. At 4 weeks after infection, there was also infiltration in the lamina propria of the body; however, the degree of infiltration of inflammatory cells was lower in the body mucosa than in the antral mucosa. These inflammatory changes increased progressively from 4 weeks to 16 weeks (Table 1). The mucosal layer of the antrum gradually thickened in comparison to the non-infected control group. The macroscopic area of swelling was consistent with that of this histological change. At 8 weeks after infection, lymphoid follicles were seen in the antral mucosa adjacent to the muscularis mucosae (Fig. 2C), but not in the body mucosa. In the antral mucosa, some haemorrhage (data not shown) and erosion (Fig. 2D) were also observed.

At 16 weeks after infection, the damage to the antrum became more severe. However, in the body, there was only mild infiltration of inflammatory cells in the lamina propria and the oxyntic glands were almost normal. Mucosal regeneration was observed in the antrum and also in the submucosa. Furthermore, a deep ulcer was also seen in the antral mucosa of two of the four infected gerbils (Fig. 3A) in which regenerated epithelium extended beyond the mucosal layer into the submucosa and the lamina muscularis mucosae was disrupted. The muscularis and the serosa were also disrupted by severe inflammatory cell infiltration. In ulcer lesions, infiltration of polymorphonuclear leucocytes was increased (Fig. 3B). These results indicated that inflammation started in the

Table 1. Inflammatory changes in the antrum and body of the gerbil stomach infected with *H. pylori* HPK127

Time after infection (weeks)	Antrum		Body	
	Submucosa	Lamina propria	Submucosa	Lamina propria
1	—	—	—	—
2	— or +	+	—	—
4	+	+	—	— or +
8	++	++	—	+
16	++ or +++	++ or +++	—	+

— (no inflammation), normal (same as non-infected control); + (mild inflammation), infiltration of polymorphonuclear leucocytes and mononuclear cells; ++ (moderate inflammation), severe infiltration of polymorphonuclear leucocytes and mononuclear cells and lymphoid follicle; +++ (severe inflammation), severe inflammation beyond subserosa with ulceration involving penetration.

*Inflammation was detected only near duodenum.

antrum, close to the duodenum, and progressed in the direction of the body; finally, the severe inflammation with *H. pylori* could lead to ulcer formation in the antral mucosa.

Treatment with amoxycillin

To verify that the inflammation was related to *H. pylori* infection in the gerbil model, the gerbils infected with *H. pylori* HPK127 were treated with 3.2 mg of amoxycillin/kg for 10 days, three times a day. Following this treatment *H. pylori* could not be recovered from the gastric mucosa (Table 2). Infiltration by inflammatory cells could not be seen (Fig. 4A). However, the numbers of *H. pylori* recovered from the gerbils treated with 0.32 mg of amoxycillin/kg were similar to those from the vehicle-treated group, and these animals had severe inflammatory cell infiltration and mucosal swelling (Fig. 4B, C). These results indicated that inflammatory cell infiltration and mucosal swelling in the gastric mucosa were directly caused by *H. pylori* colonisation.

Discussion

The present study demonstrated for the first time that *H. pylori* could cause ulcers in the Mongolian gerbil which extend beyond the submucosa and muscularis layer and reach the serosa in the antral mucosa in two of four animals tested at 16 weeks after infection. These data indicate that *H. pylori* infection was related directly to ulcer formation in the gastric mucosa. Previously, no direct evidence has been reported on the ulcerogenicity of *H. pylori* in man and in experimental animal models, except the report of a relationship between ulcer and *H. pylori* in gnotobiotic piglets in which small and superficial ulcers occurred in the stomach [19]. Speculation about the relationship between *H. pylori* and ulcer was based only on the data that ulcer recurrence was reduced after eradication of *H. pylori* [3]. From the data presented here, the Mongolian gerbil model can contribute to the study of *H. pylori*-induced ulcers.

In this Mongolian gerbil model, severe infiltration by polymorphonuclear leucocytes and mononuclear cells in the mucosal lamina propria and submucosa of the antrum were observed 4 weeks after infection and

Fig. 2. Severe gastritis in the antrum mucosa of Mongolian gerbils infected with *H. pylori* HPK127. **A**, antral mucosa of non-infected gerbil (bar = 100 μ m). **B**, mononuclear cell infiltration in the antral mucosa adjacent to the muscularis mucosae at 2 weeks after infection (bar = 100 μ m). **C**, lymphoid follicle in the antral mucosa at 8 weeks after infection (bar = 200 μ m). **D**, erosion in the epithelial layer at 8 weeks after infection (bar = 100 μ m).

Fig. 3. Ulceration in the antrum of Mongolian gerbils at 16 weeks after infection with *H. pylori* HPK127. **A**, deep ulcer in the antral mucosa (bar = 400 µm). **B**, many polymorphonuclear cells can be seen in the ulcer lesion (bar = 40 µm).

Table 2. Recovery of *H. pylori* from the antral and body mucosa of Mongolian gerbils treated with amoxycillin

Amoxycillin dose (mg/kg)	Macroscopic observation	Mean (SD) log ₁₀ cfu/mucosal sample		Frequency of clearance
		Antrum	Body	
3.2	—	< 2	< 2	4/4
0.32	+	5.59 (0.39)	3.22 (0.46)	0/4
0	+	5.54 (0.21)	3.24 (0.74)	0/4

Amoxycillin was suspended in methyl cellulose solution 0.5%; —, normal (same as non-infected control); +, swelling of the antral mucosa.

lymphoid follicles in the antral mucosa at 8 weeks. These are common features of *H. pylori*-associated gastritis [20]. These histopathological changes increased rapidly in severity from 4 weeks to 16 weeks after infection and progressed to the body mucosa. These results indicate that inflammatory changes in gerbils may progress more rapidly than those in man and other small animals. On the other hand, the severity of inflammation in the oxyntic gland increased very slowly in comparison to the antrum and severe gastritis such as lymphoid follicles may take a long time to develop. This suggests that the aetiological difference between gastritis in the antrum and

the body mucosa may primarily depend on the colonising efficiency of *H. pylori* in the antrum and the body (Fig. 1, Table 1), and that the severity of inflammation was related to the number of colonising *H. pylori* [21, 22].

There are some animal models for *H. pylori* in terms of the occurrence of severe gastritis [9, 10, 23]. Severe inflammatory cell infiltration and lymphoid follicles were observed in the gastric mucosa in gnotobiotic piglet, dog and non-human primate models infected with *H. pylori* isolated from man, and specific pathogen-free cats infected with *H. pylori* from the

Fig. 4. Histopathological examination of *H. pylori*-infected Mongolian gerbils treated with amoxycillin (bar = 100 μ m). **A**, antral mucosa of a gerbil treated with amoxycillin (3.2 mg/kg). **B**, antral mucosa of a gerbil treated with amoxycillin (0.32 mg/kg). **C**, antral mucosa of vehicle-treated gerbils.

cat. Small animals such as mice are the most useful experimental animals. However, infection by *H. pylori* isolated from man causes only mild gastritis in the gastric mucosa of established small animal models [24]; so adaptation to animals is required to cause active gastritis [11]. However, inflammation is still insufficient; some genetic exchange or alteration of virulence factors may occur during adaptation to animals. On the other hand, severe histopathological changes were found in mouse models infected with

the *H. pylori*-like organisms, *H. felis* and *G. hominis* (*H. heilmannii*) [12, 25]. Therefore, these models have contributed to the histological study of *H. pylori*. However, unknown virulence factors in these species, which are not present in *H. pylori*, may have caused the severe gastritis in mouse gastric mucosa. Therefore, it is necessary to demonstrate that severe gastritis and ulcers in animal models can be induced by *H. pylori* isolates from man. Ulcers and gastritis in Mongolian gerbils that were infected with *H. pylori* isolates from man were more severe than in the previous established models of *H. pylori* infection and this model is more suitable for studying the pathogenesis of *H. pylori* infection.

To verify that the gastritis was caused by *H. pylori*, the gerbils were treated with amoxycillin. Clearance of the organisms from the antral and body mucosa was followed by the disappearance of inflammatory cells from the lamina propria and submucosa. This indicates that *H. pylori* directly caused severe inflammatory changes in the gastric mucosa of Mongolian gerbils and may also induce ulcers.

Yokota *et al.* [26] first reported a Mongolian gerbil model for *H. pylori* infection. However, *H. pylori* could not be recovered from the gastric mucosa and only a few inflammatory cells were observed. Recently, severe inflammation was demonstrated in the gastric mucosa of Mongolian gerbils infected with a standard strain of *H. pylori* (ATCC43504) [27]. These data were insufficient to show the differences in inflammation and colonisation of *H. pylori* between the antrum and the body of the stomach of Mongolian gerbils. No report on the ulcerogenicity of *H. pylori* in Mongolian gerbils has been presented previously.

The reasons why gerbils are susceptible to *H. pylori* are not clear. Connective tissue mast cells (CTMC) instead of mucosal mast cells (MMC) are present in the jejunum of gerbils, although MMC are usually observed in the mucosal lamina propria in mouse and rat [28]. MMC are considered to be effector or regulator cells of immune-mediated expulsion of certain intestinal parasites [29]. If CTMC are unable to eliminate *H. pylori* as efficiently as MMC, then the organism can grow easily and cause severe gastritis in the gastric mucosa of gerbils. However, other factors such as cytokine production or adhesion molecules cannot be excluded.

In conclusion, Mongolian gerbils infected with *H. pylori* causing ulceration and severe gastritis is one of the best animal models for helping to understand the pathogenesis of *H. pylori* infection and to evaluate therapy.

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