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Experimental Gastritis Induced by *Helicobacter pylori* in Japanese Monkeys

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We used Japanese monkeys (*Macaca fuscata*) to establish an experimental model in order to clarify the pathogenicity of *Helicobacter pylori* in gastric and duodenal disorders. A suspension (5 ml; 10^9 CFU/ml) of *H. pylori* cells isolated from humans was sprayed around the antrum of the stomach of each of 12 of 17 animals with an endoscope. The remaining five animals were not inoculated; they served as a control group. On days 7, 14, and 28 after inoculation, the gastric mucosa samples were examined grossly and were biopsied for microscopic examination with an endoscope. *H. pylori* was recovered from 7 of the 12 inoculated animals (58%), and infiltration by neutrophils and monocytes was observed histologically. Macroscopic gastritis with erythema and erosions were noted for five of these animals. On day 28 after inoculation, five animals in the infected group were treated with ampicillin. In two infected but untreated animals, the bacteria persisted for more than 6 months. The result of the gastritis scoring of the antral mucosa and the ammonia concentration in the gastric secretion were significantly higher ($P < 0.01$ to 0.001) for the infected group than for the control group; however, these values decreased to levels comparable to those for the control group after treatment with ampicillin. Urease activity was positive in gastric biopsy specimens from five of the seven animals in the infected group after 7 days and from four of these animals after 14 days but was negative in all specimens from animals in the control group. The level of antibody (immunoglobulin G) in serum for the infected group was elevated but changed very little for the control group. These results suggest that this *M. fuscata* model can be used to study *H. pylori* infection and that *H. pylori* can induce gastritis.

The involvement of *Helicobacter pylori* cells in the pathogenesis of gastritis and duodenal and gastric ulcers and their epidemiology and treatment have been reported by a number of investigators (10, 14, 15, 22) since 1983, when Warren and Marshall (23) first isolated "unidentified curved bacilli," later designated *H. pylori* (7), from human gastric mucosae. This bacterium is often isolated from the antral mucosae of patients with gastritis and peptic ulcer (8, 15, 16, 22). It causes acute gastritis and subsequent chronic gastritis in human volunteers (14, 17); however, neither the pathogenesis of the disease caused by *H. pylori* nor its optimum antibacterial therapy is known. One reason for the delay in progress in this field is that only a few species of animals are capable of being infected by this bacterial species. Small animals such as mice, rats, rabbits, and guinea pigs have been orally inoculated with *H. pylori* in attempts to induce gastritis, but such attempts have been unsuccessful (11, 21).

Recently, gastritis similar to that found in humans has been established in gnotobiotic piglets (2, 11, 12), barrier-born pigs (3), gnotobiotic beagle dogs (21), and rhesus monkeys (4, 6) after inoculation with *H. pylori*, demonstrating that it is possible to produce models of infection with this bacterium.

We sought to determine whether *H. pylori* can survive in the gastric mucosa of the Japanese monkey (*Macaca fuscata*), which is readily available in Oita, Japan, and whether infection in these animals resembles that in humans. Our findings both suggest that the monkey *M. fuscata* can be used as a model of *H. pylori* infection and provide evidence that *H. pylori* is pathogenic in the gastric mucosa.

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MATERIALS AND METHODS

Animals. Wild Japanese monkeys (*M. fuscata*) were given food designed for them (Oriental Yeast Co., Tokyo, Japan), as well as tap water, and were housed in separate cages at the Animal Laboratory Center of Oita Medical University. A total of 17 animals were examined endoscopically, histologically, and bacteriologically before study. All of these monkeys, which were free of *H. pylori* infection, were included in this study (Table 1). The present study was conducted in accordance with Oita Medical University guidelines for animal experimentation.

Bacterial strains. The bacterial strains used were *H. pylori* MCO 88155, MCO 88099, MCO 88142, and MCO 88156 isolated from two patients with gastric ulcers and two patients with duodenal ulcers. The bacterial strains were identified as *H. pylori* if they were microaerobic, gram-negative, curved rods; oxidase positive; catalase positive; urease positive; nitrate reductase negative; resistant to nalidixic acid; and sensitive to cephalothin (15, 19).

Isolation of bacteria from gastric biopsy specimens. Biopsy specimens were placed in containers containing 2 ml of sterile 20% glucose solution, transported to the laboratory within 2 h, smeared on 7% sheep blood agar plates (basic medium, heart infusion agar; BBL Microbiology Systems, Cockeysville, Md.) and Belo-Horizonte medium (20), and cultured under microaerobic conditions in anaerobic jars (Campypak System; BBL Microbiology Systems) at high humidity and 37°C for 4 days. The isolated bacterial strains were identified and stored in sterile 10% skim milk solution at −80°C.

Preparation of bacterial inoculum. The bacterial strains stored at −80°C were thawed at room temperature and cultured on 7% sheep blood agar plates under microaerobic

TABLE 1. Animals used in this study[a]

Animal group	Estimated age (yr)	Body wt (kg)	Sex,[b] no.
Inoculation challenged (n = 12)	13.3 ± 2.9	11.5 ± 4.6	M, 3; F, 9
Non-inoculation challenged (n = 5)	10.4 ± 1.5	8.4 ± 1.1	M, 1; F, 4

[a] Values are means ± standard deviations.
[b] M, male; F, female.

conditions at 37°C for 4 days. The resulting colonies were suspended in 5 ml of sterile saline, and the bacterial concentration was adjusted to 10^9 CFU/ml with a spectrophotometer (UV-120-01; Shimadzu, Kyoto, Japan) calibrated in advance for counting bacteria. Two-milliliter aliquots of each of the four bacterial suspensions were resuspended in 8 ml. Five milliliters of the final resuspensions was used in each monkey.

Inoculation of animals. The animals were given ampicillin dry syrup (Meiji-Seika Co. Ltd., Tokyo, Japan) (30 mg/kg of body weight orally) for 14 days to eradicate spiral bacteria in the stomach. Sodium bicarbonate (1 g/day orally) and famotidine (Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan) (20 mg/kg intramuscularly) were given for 3 days prior to challenge with the bacterial suspension. Food was withheld from the animals for 48 h before bacterial inoculation; only tap water was given. Twelve animals were anesthetized with ketamine hydrochloride (Ketalar; Sankyo Co., Ltd., Tokyo, Japan), and a 5-ml suspension of *H. pylori* was sprayed endoscopically (GIF-P3; Olympus Co., Ltd., Tokyo, Japan) around their antra via a tube. The remaining five animals were not inoculated and served as the control group.

Stomach biopsy specimens. The gastric mucosa was examined endoscopically, and biopsy specimens were collected from the antrum within 3 cm of the pyloric ring before inoculation and 7, 14, and 28 days after inoculation both from the infected and from the control groups. Each biopsy specimen was cultured for *H. pylori* and examined microscopically after hematoxylin and eosin staining for the gastritis score, Warthin-Starry silver staining, and Gram staining for examination of spiral bacteria.

Rapid urease test. The rapid urease test was performed by modifying the method described by Arvind et al. (1). Urea (Wako Pure Chemical Industries Ltd., Tokyo, Japan) and phenol red were dissolved in distilled water to final concentrations of 10 and 0.01%, respectively, and the pH of the solution was adjusted to 6.0 with 0.1 M sodium dihydrogen phosphate. The solution was sterilized by passage through a millipore filter (0.22-μm pore size; Nihon Millipore Kogyo, Tokyo, Japan), aliquoted (0.5 ml) into 3-ml vials, and stored at 4°C. One biopsy specimen was placed in the test solution immediately after collection and was determined to be positive for urease if its color changed from pale yellow to red within 120 min or negative if no color change was observed.

Evaluation of gastritis. The grade of gastritis was evaluated by a scoring system based on the method described by Rauws et al. (22). The maximum total score was 10, and gastritis was considered more severe at higher scores.

Ammonia concentration in gastric secretions. Gastric secretion samples (4 to 5 ml each) were collected before endoscopic biopsy. The ammonia concentrations were measured by a modification of the Okuda-Fujii method (18).

Serum anti-*H. pylori* antibody. Blood (5 ml) was drawn before inoculation with the *H. pylori* suspension and 14 and 49 days after inoculation (also after 3 and 6 months for the two infected but untreated animals). Blood from the control group was similarly sampled. The serum was separated, and specific serum immunoglobulin G (IgG) was measured by enzyme-linked immunosorbent assay (ELISA) with lysates of the standard strain (*H. pylori* NCTC 11639) and the four strains used in this study in 0.2 M glycine-hydrochloride buffer (pH 2.2) as antigens and peroxidase-labeled anti-monkey IgG antibody (Cappel, Durham, N.C.) on the basis of the method described by Goodwin et al. (9).

Eradication of *H. pylori*. Ampicillin dry syrup (MIC required for *H. pylori*, <0.1 μg/ml) was administered orally to five of the seven animals infected by *H. pylori* in a dose of 30 mg/kg once every day for 21 days, beginning on day 28 after inoculation. After treatment with ampicillin, biopsy specimens were collected endoscopically from the gastric mucosae, *H. pylori* was isolated, the gastritis scores were evaluated, and the ammonia concentrations in the gastric secretions and levels of antibody in the serum samples were determined to assess the effects of ampicillin administration. The remaining two animals in the infected but untreated group were also examined 3 and 6 months after inoculation.

Statistical analysis. Statistical analysis was performed by Student's t test.

RESULTS

Colonization by *H. pylori*. Colonization of the gastric mucosa by *H. pylori* was observed in 7 of the 12 animals inoculated with the bacterium (Table 2). *H. pylori* was recovered from the gastric biopsy specimens from six animals by culture. Spiral bacteria were detected histologically in nine animals, and the rapid urease test gave a positive result for five animals seven days after inoculation. After 14 days, *H. pylori* was detected in seven animals by both culture and microscopic examination, and the rapid urease test gave a positive result for four animals. On day 28 after inoculation, bacteria in all seven animals (no. 1 to 7) were demonstrated by both culture and microscopy and the rapid urease test gave a positive result for four animals. No bacteria were recovered from 5 of the 12 animals. For the control group, no *H. pylori* was isolated from stomach biopsy specimens, no spiral bacteria were observed microscopically, and the rapid urease test gave a negative result for all five animals.

Endoscopic examination of gastric mucosa lesions. (i) Gross findings. Endoscopy findings 7 days after inoculation showed macroscopic gastritis accompanied by antral erosions and erythema in 5 animals (Table 2). Similar findings were obtained for four animals at 14 days but had disappeared by 28 days. No macroscopic gastritis in the five animals in which *H. pylori* did not colonize was noted.

(ii) Histological findings. Figures 1 and 2 show changes in the gastritis score and histological findings. The antral gastritis scores of the five animals infected with *H. pylori* were 1.4 ± 0.6 (mean ± standard deviation) before inoculation but increased significantly ($P < 0.001$) to 6.6 ± 0.5 on day 7 and to 6.6 ± 0.8 on day 14 after inoculation with *H. pylori*. Infiltration by inflammatory cells such as polymorphonuclear leukocytes and monocytes was also noted (Fig. 2B). The score was reduced slightly to 3.6 ± 0.2 after 28 days, but infiltration by lymphocytes and plasma cells persisted.

After treatment with ampicillin, the gastritis scores decreased significantly ($P < 0.001$) to 1.6 ± 0.2, with improvement in inflammatory-cell infiltration (Fig. 2C). The gastritis

TABLE 2. Results of cultures, microscopic examinations for curved bacilli, and rapid urease tests and endoscopic evidence of macroscopic gastritis

Monkey group and no. (sex)	Test results before or at following times after inoculation[a]:						
	Before	7 days	14 days	28 days	49 days	3 mo	6 mo
Inoculation challenged (n = 12)[b]							
1 (M[c])	−/−/−/−	+/+/+/+	+/+/+/+	+/+/+/−	−/−/−/−	NT[d]	NT
2 (F[c])	−/−/−/−	−/+/−/−	+/+/−/−	+/+/+/−	−/−/−/−	NT	NT
3 (F)	−/−/−/−	+/+/−/+	+/+/−/−	+/+/+/−	−/−/−/−	NT	NT
4 (F)	−/−/−/−	+/+/+/+	+/+/−/+	+/+/−/−	−/−/−/−	NT	NT
5 (M)	−/−/−/−	+/+/+/−	+/+/+/−	+/+/−/−	−/−/−/−	NT	NT
6 (F)	−/−/−/−	+/+/+/+	+/+/+/+	+/+/+/−	+/+/+/−	+/+/−/−	+/+/−/−
7 (F)	−/−/−/−	+/+/+/+	+/+/+/+	+/+/−/−	+/+/−/−	+/+/+/−	+/+/+/−
8 (F)	−/−/−/−	−/−/−/−	−/−/−/−	NT	NT	NT	NT
9 (F)	−/−/−/−	−/+/+/−	−/−/+/−	NT	NT	NT	NT
10 (M)	−/−/−/−	−/−/−/−	−/−/−/−	NT	NT	NT	NT
11 (F)	−/−/−/−	−/+/−/−	−/−/−/−	NT	NT	NT	NT
12 (F)	−/−/−/−	−/−/−/−	−/−/−/−	NT	NT	NT	NT
Non-inoculation challenged (control [n = 5])							
13 (F)	−/−/−/−	−/−/−/−	−/−/−/−	−/−/−/−	NT	NT	NT
14 (F)	−/−/−/−	−/−/−/−	−/−/−/−	−/−/−/−	NT	NT	NT
15 (M)	−/−/−/−	−/−/−/−	−/−/−/−	−/−/−/−	NT	NT	NT
16 (F)	−/−/−/−	−/−/−/−	−/−/−/−	−/−/−/−	NT	NT	NT
17 (F)	−/−/−/−	−/−/−/−	−/−/−/−	−/−/−/−	NT	NT	NT

[a] Results (positive [+] or negative [−]) are given in the following order: isolation of *H. pylori*; detection of microscopic curved bacilli; rapid urease test; evidence for macroscopic gastritis.

[b] A 5-ml suspension of *H. pylori* (10^9 CFU/ml) was sprayed around the antrum of the stomach with an endoscope. Ampicillin was administered orally to five animals infected with *H. pylori* (no. 1 to 5) at 30 mg/kg/day for 21 days, beginning at day 28 after inoculation. The remaining two infected animals (no. 6 and 7) were monitored without treatment.

[c] F, female; M, male.

[d] NT, not tested.

scores were significantly lower and inflammatory-cell infiltration results were also less for the control group than for the infected group from 7 to 28 days after inoculation.

Ammonia concentration in gastric secretions. In five animals in the infected group, the ammonia concentrations in gastric secretions were 2,984 ± 699 μg/dl before inoculation with *H. pylori* but increased significantly to 12,381 ± 3,770 μg/dl ($P < 0.01$) after 7 days, 9,746 ± 1,719 μg/dl ($P < 0.001$) after 14 days, and 9,649 ± 1,302 μg/dl ($P < 0.001$) after 28 days. After treatment with ampicillin, the concentrations decreased significantly ($P < 0.001$) to 2,882 ± 821 μg/dl. For the control group, no significant change was observed in the ammonia concentration, which was significantly lower than it was for the infected group at 7 ($P < 0.025$), 14 ($P < 0.01$), or 28 ($P < 0.01$) days after inoculation (Fig. 3).

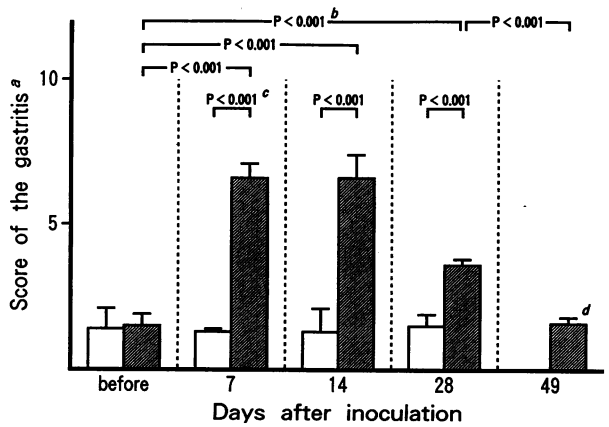
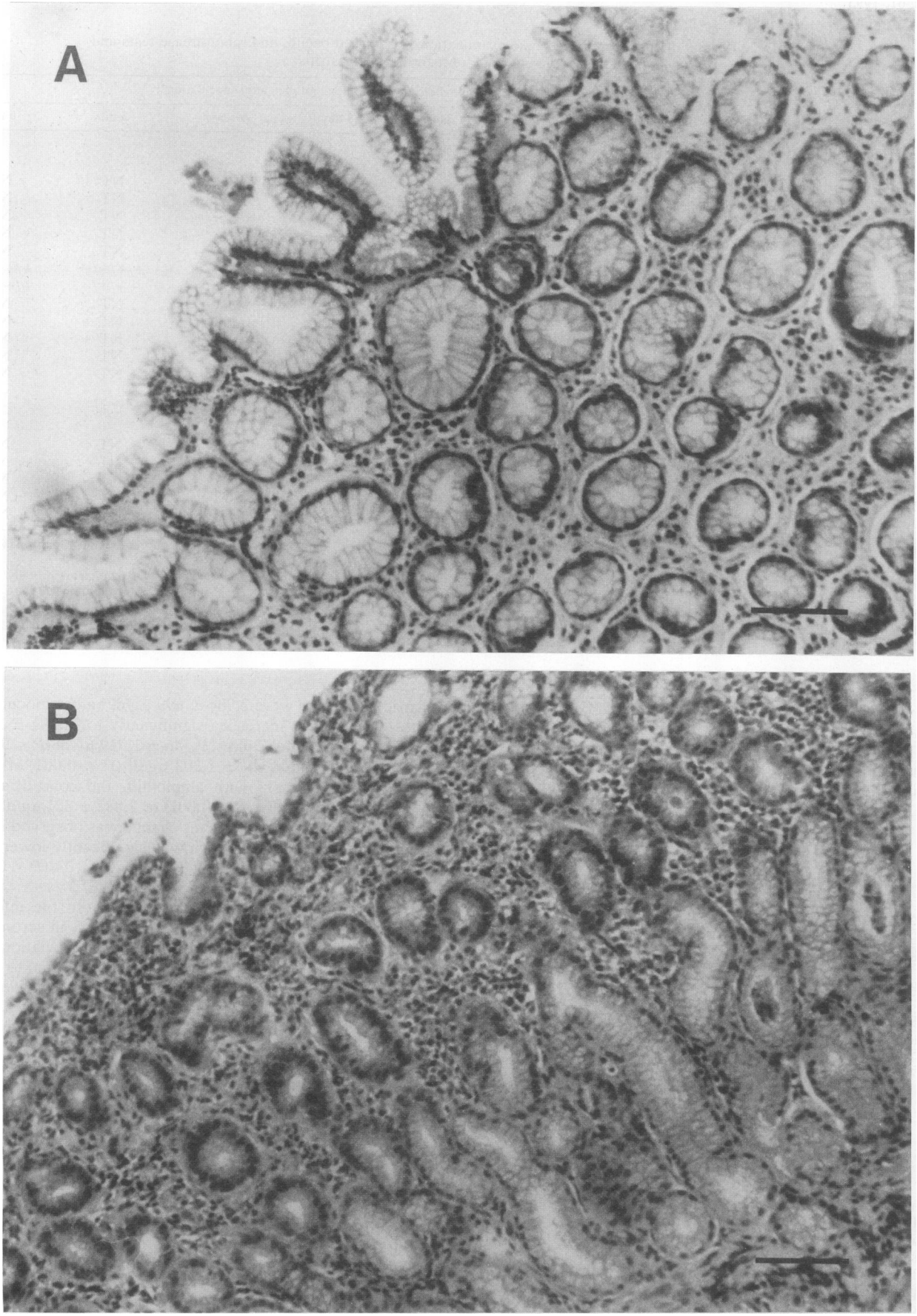


FIG. 1. Changes in the gastritis score in Japanese monkeys. *a*, see the text for definition of the gastritis score. *b*, in the infected group, significantly high scores ($P < 0.001$) were observed at 7, 14, and 28 days after inoculation, compared with those for the control group. *c*, difference between the infected group and the control group was not significant before inoculation but was significant ($P < 0.001$) 7, 14, and 28 days after inoculation. *d*, value after treatment with ampicillin for 21 days, beginning at 28 days after inoculation. Empty box, control group (n = 5); striped box, infected group (n = 5).

Changes in levels of antibody in serum. In the infected group (five animals), the level of IgG antibody in serum was 64.6 ± 24.2 ELISA units (EU) before inoculation and increased slightly to 96 ± 55.8 EU after 14 days and to 153.6 ± 101.8 EU after 49 days, although the difference was not significant. In the control group, it remained between 52.6 ± 0.9 EU and 62.6 ± 2.6 EU, showing no marked change (Fig. 4).

Findings after antibiotic treatment. No *H. pylori* in cultures from any of the five infected animals was recovered, and both the gastritis score and the ammonia concentration in gastric juice decreased significantly after ampicillin treatment (Fig. 1 and 3). However, the level of antibody in serum tended to increase even after treatment (Fig. 4).

Findings in two infected animals after 6 months. As shown in Table 2 and Fig. 5, in two animals (no. 6 and 7), bacteria were detected by culture and histological examination, and macroscopic gastritis was observed 7 days after inoculation. After 28 days, however, the gastritis became obscure and rapid urease test results were inconsistent. Infiltration by monocytes and polymorphonuclear leukocytes was noted in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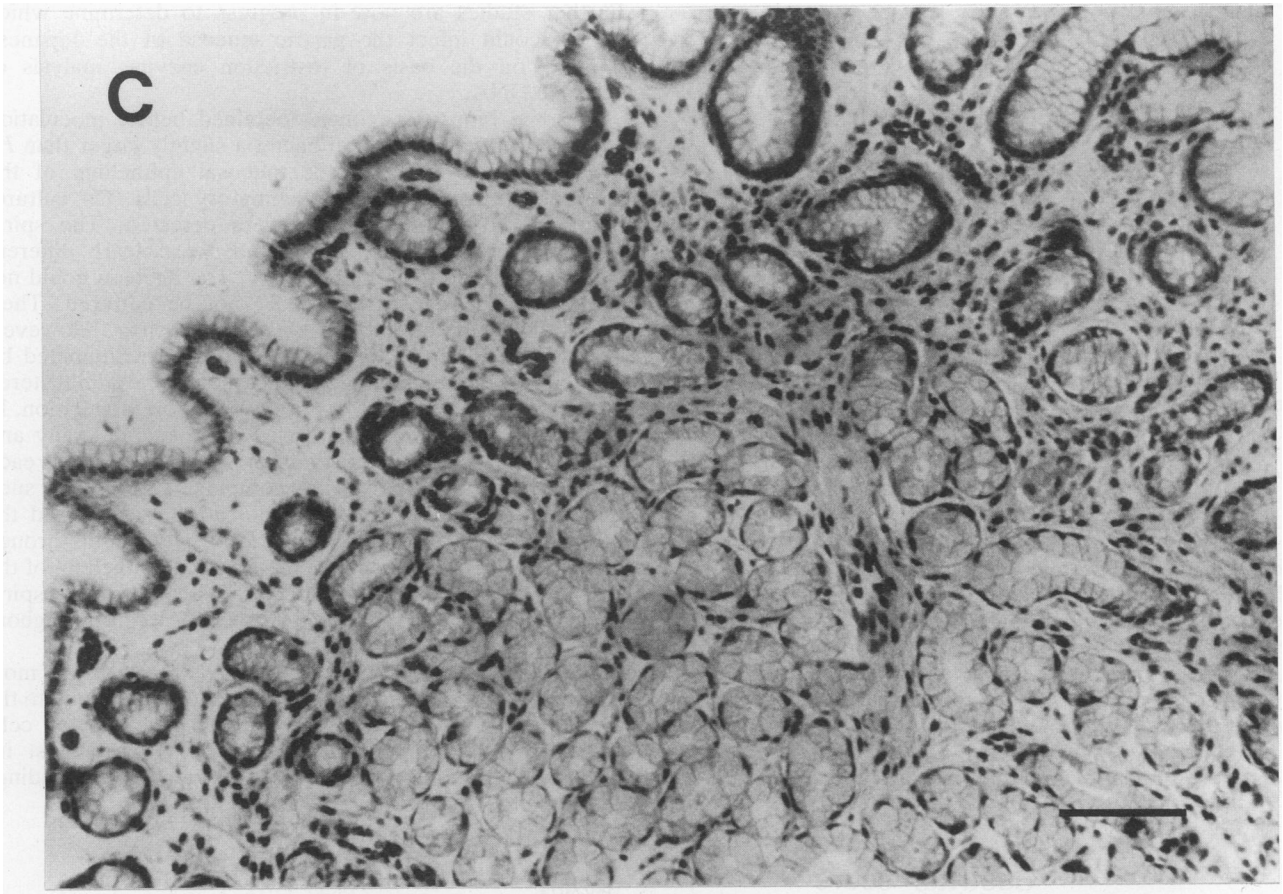

FIG. 2. Histological findings in the antral mucosae of Japanese monkeys infected with *H. pylori* (hematoxylin and eosin stain; bar, 100 μm; magnification, ×94). (A) Before inoculation with *H. pylori*. Inflammatory-cell infiltration of the lamina propria of the stomach was unremarkable. (B) At 14 days after inoculation with *H. pylori*. Monocytes and polymorphonuclear leukocyte infiltration in the lamina propria were evident. Inflammatory-cell infiltration in the mucosal epithelium and superficial erosions were noted. (C) After administration of ampicillin for 21 days, beginning at 28 days after inoculation with *H. pylori*. Inflammatory-cell infiltration of the lamina propria was unremarkable; only a few monocytes could be observed.

the gastric mucosa 7 and 14 days after inoculation and persisted until after 28 days; however, the polymorphonuclear leukocytes nearly disappeared after 3 months, leaving monocytes as the primary cell infiltrate. The gastritis score decreased after 6 months, but neither cell infiltration nor the gastritis score had decreased to control levels. The ammonia concentration in gastric secretions remained high after inoculation, and the level of antibody (IgG) in serum tended to increase gradually from the level measured before inoculation.

DISCUSSION

Experimental animal models of *H. pylori* infection are indispensable in clarifying the pathogenic significance of this bacterium in gastritis and peptic ulcers. Gnotobiotic piglets, barrier-born pigs, gnotobiotic beagle dogs, and rhesus monkeys are considered sensitive to *H. pylori*, and inoculation challenge tests with *H. pylori* have been performed with these animals (2–6, 11, 13, 21).

In our initial animal experiments, we endoscopically administered 5 ml of a bacterial suspension (10^6 CFU/ml) mixed with four strains of *H. pylori* isolated from humans; however, no infection could be produced. Bacterial coloni-

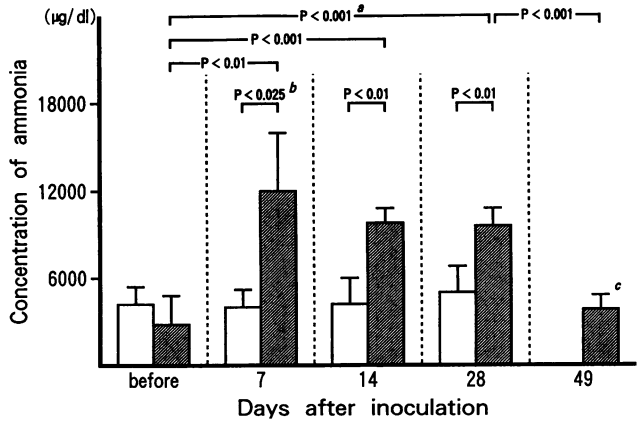

FIG. 3. Changes in gastric ammonia concentration in Japanese monkeys. *a*, for the infected group, significant differences were observed between the value before inoculation and the values 7 ($P < 0.01$), 14 ($P < 0.001$), and 28 ($P < 0.001$) days after inoculation. *b*, difference between the infected group and the control group was not significant before inoculation but was significant 7 ($P < 0.025$), 14 ($P < 0.01$), and 28 ($P < 0.01$) days after inoculation. *c*, value after ampicillin treatment for 21 days, beginning on day 28 after inoculation. Empty box, control group ($n = 5$); striped box, infected group ($n = 5$).

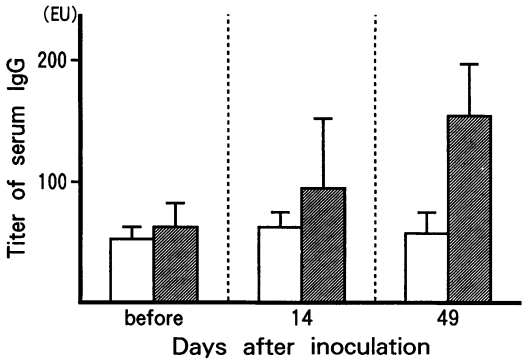

FIG. 4. Changes in the levels of antibody (IgG) in the sera of Japanese monkeys. There was no significant difference between the IgG level in serum before inoculation and the level measured 49 days after inoculation. Empty box, control group (n = 5); striped box, infected group (n = 5).

zation was observed when the inoculum size was increased to 10^9 CFU/ml; at this inoculum size, gastritis accompanied by erosions was produced in the antrum. This suggested that bacteria are not likely to colonize the gastric mucosa unless the *H. pylori* inoculum size is at least greater than 10^7 CFU/ml. In the present study, a mixed suspension of four *H. pylori* strains isolated from patients was inoculated, because the aim of the present experiment was to establish an animal model for studies of *H. pylori*-related human diseases. Further studies are now in progress to determine which strains could infect the gastric mucosa of the Japanese monkey on the basis of restriction enzyme analysis of genomic DNA.

In some biopsy specimens obtained before inoculation with *H. pylori*, a few spiral bacteria slightly larger than *H. pylori* were observed in the mucosal epithelium of the stomach with just a few inflammatory cells. On culture, however, no *H. pylori* cells were detected. The spiral bacteria observed in the present study were clearly different from *H. pylori* cells in morphology. The bacteria could not be identified because they could not be cultured. Their susceptibility to antibiotics was also unclear. However, since these spiral bacteria were completely eliminated by ampicillin treatment in a preliminary study, we administered ampicillin orally for 2 weeks prior to *H. pylori* inoculation. In addition, sodium bicarbonate was administered orally and famotidine was administered intramuscularly for 3 days each to increase the pH of the gastric contents. As a result of such treatment, the spiral bacteria that naturally colonized the animals were eliminated and *H. pylori* easily passed through the acidic barrier and reached the mucosal epithelium of the stomach. After *H. pylori* infection developed, no spiral bacteria different from *H. pylori* were observed throughout this experiment.

The macroscopic gastritis induced by *H. pylori* was most prominent in the antrum and was milder from the body to the fornix of the stomach. Histologically, inflammatory cells (including polymorphonuclear leukocytes) were most intense in the lamina propria of the antrum. These findings

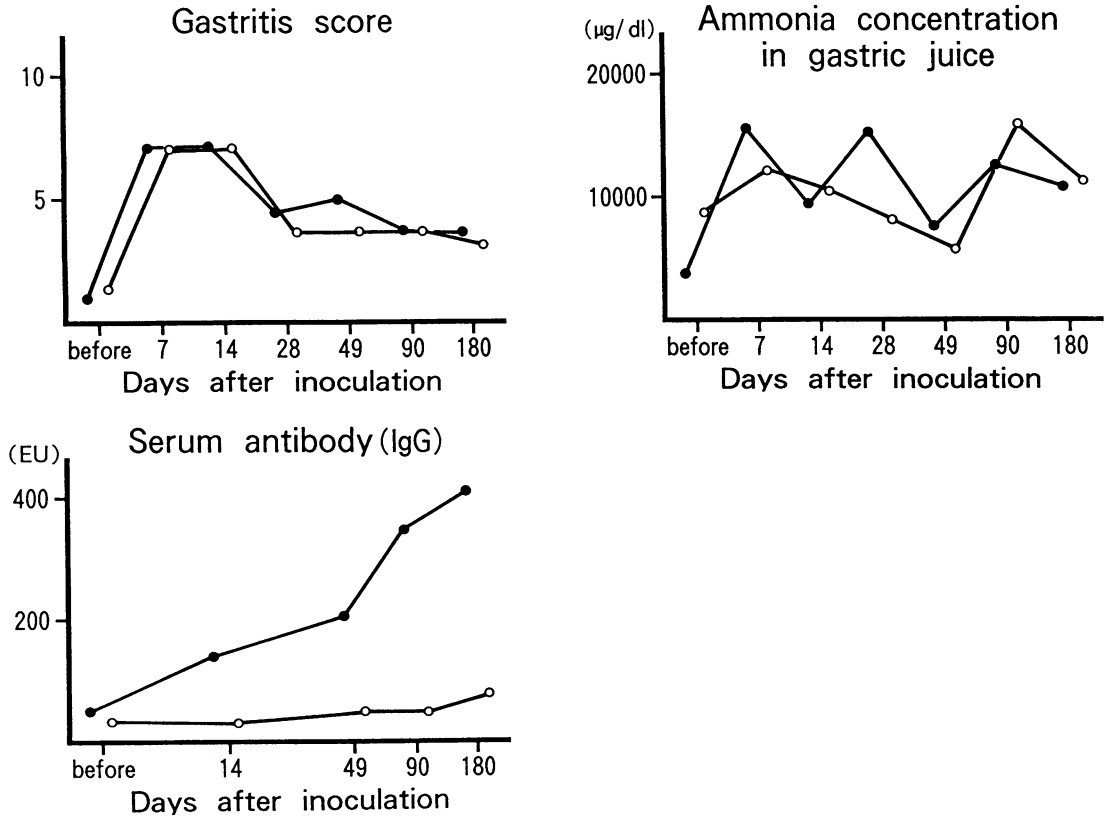

FIG. 5. Results for two infected monkeys after 6 months of observation without treatment. ○, changes in Japanese monkey no. 6; ●, changes in Japanese monkey no. 7.

persisted from 7 to 14 days after inoculation and are consistent with acute gastritis, but no macroscopic gastritis could be detected after 28 days. In the two animals that were observed for 6 months, infiltration of the antral mucosa by polymorphonuclear leukocytes decreased within 3 to 6 months, and the remaining inflammatory cells consisted mostly of monocytes, such as lymphocytes and plasma cells; however, inflammatory-cell infiltration persisted at a level higher than that before inoculation. These histological findings for the gastric mucosa were similar to the results of earlier animal experiments (2–6, 11, 13, 21) and nearly identical to findings for the gastric mucosae of *H. pylori*-positive patients with chronic active gastritis (16, 22). The antibody levels in the two monkeys were different from one another; the reason for this is unknown. These two *H. pylori*-infected monkeys have now been under observation without any treatment for more than 18 months, and *H. pylori*-associated gastritis has continued.

To obtain supporting evidence of *H. pylori* infection, bacterial urease activity was evaluated by the rapid urease test. This test provides an index of the concentration of the bacteria. We also determined the concentration of ammonia produced by the bacterial urease in the gastric secretions. In Japanese monkeys infected with *H. pylori*, the gastric mucosae were positive for urease and the concentrations of ammonia in gastric juice were significantly increased. These values decreased significantly and pathologic changes in the antral mucosae improved markedly after treatment with ampicillin. For the control group, no significant change in these values was observed. Moreover, the level of antibody to *H. pylori* in serum increased gradually, providing further solid evidence of established *H. pylori* infection. These results suggest that the Japanese monkey (*M. fuscata*) can be used as an experimental model of *H. pylori* infection and that *H. pylori* can cause gastritis.

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