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Helicobacter pylori Gastric Infection in Gnotobiotic Beagle Dogs

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Establishment of infection with *Helicobacter pylori* and gastritis in nonhuman species is currently only successful in gnotobiotic piglets. This study was designed to determine whether *H. pylori* will colonize the gastrointestinal tract of gnotobiotic dogs. Gnotobiotic beagle pups were derived by standard methods. Group A (five dogs) was orally challenged with 3×10^8 *H. pylori* at 7 days of age. Group B (two dogs) received only peptone water but was contact-exposed beginning on day 23 postinfection (p.i.). Necropsy was performed on dogs on day 30 p.i. *H. pylori* colonized the stomach of all dogs (groups A and B). Urease map analysis correlated with the microbiologic findings and indicated that the density of colonization was less than that observed in human tissue. Organisms were also recovered from the pharynx, esophagus, duodenum, and rectum of 1, 2, 2, and 1 dog, respectively. All group A and one group B dog developed serum immunoglobulin G specific for *H. pylori* by day 30 p.i. Gross lesions were restricted to the stomach and consisted of small (<1 mm) lymphoid follicles. Microscopically, there were focal to diffuse lymphoplasmacytic infiltrates with follicle formation and mild to moderate infiltration of neutrophils and eosinophils in the gastric lamina propria. With the Warthin-Starry silver stain, organisms were seen on the surface of the gastric epithelial cells, beneath the mucus layer. We conclude that *H. pylori* colonizes the stomachs of gnotobiotic dogs for at least 1 month and the lesions resemble those seen in humans. *H. pylori* is transmissible by contact from infected to noninfected dogs.

Helicobacter pylori (formerly *Campylobacter pylori*) is a gram-negative microaerophilic bacterium that causes gastritis and is associated with nonulcer dyspepsia and gastroduodenal ulcer in humans (6, 18). Oral challenge of volunteers results in histologic lesions of chronic active gastritis as well as symptoms of dyspepsia (19, 20). Treatment of *H. pylori*-associated gastritis with combinations of antimicrobial agents, bismuth, and H-2 antagonists in humans has met with limited success, and recurrence is frequent (10). Because of this, the use of human volunteers to study this disease entity is not practical, and adequate models for study of the pathogenesis of this disease are needed.

H. pylori will not colonize many of the usual laboratory animal species, including conventionally reared rats, mice, rabbits, guinea pigs, specific-pathogen-free pigs, colostrum-deprived piglets, and gnotobiotic rats and mice (9; Krakowka et al., Second Int. Symp. on *C. pylori*, in press). We and others have shown that the gnotobiotic neonatal piglet is susceptible to oral infection with *H. pylori* (15, 16). In this model, infection is limited to the stomach, and the lesions that develop are those of lymphoplasmacytic gastritis, resembling human infection (5). The gnotobiotic pig model, however, is limited because of the inability to maintain pigs in the gnotobiotic state for greater than 45 to 60 days because of size and nutritional constraints. In addition, study of ulcerogenesis may be confounded by the susceptibility of weanling pigs to the development of ulcers induced by diet and stress (3). The advantages of a dog model include the ability to maintain the animals in the gnotobiotic condition for years, the availability of well-established methods for studying immunologic and gastric physiologic responses, and the lack of propensity to develop spontaneous ulcers. The objective of this study was to determine whether gnotobiotic dogs are susceptible to gastric infection by *H. pylori*.

MATERIALS AND METHODS

Animals. A litter of seven gnotobiotic beagle pups was derived from specific-pathogen-free bitches by standard methods (14). They were maintained in sterile Pentub isolation units and fed a diet of Esbilac (PatAg, Inc., Hampshire, Ill.). Initially, unchallenged control dogs were housed separately from inoculated dogs. Beginning on day 23 postinfection (p.i.), controls were housed together with infected dogs to determine whether infection would spread via contact.

Bacterial inoculum. A virulent strain of *H. pylori*, 26695, was used. This is a human isolate and is capable of colonizing and producing gastritis in gnotobiotic piglets (7). Bacteria were grown in 250-ml Erlenmeyer flasks containing brucella broth (Difco Laboratories, Detroit, Mich.) supplemented with 10% fetal calf serum (BBL Microbiology Systems, Cockeysville, Md.). Flasks were incubated at 37°C in a 10% CO_2 atmosphere on a rotary shaker at 150 rpm. Cultures were harvested by centrifugation 24 h after inoculation (logarithmic growth phase), washed, and suspended in peptone water. Organisms were enumerated with a hemacytometer and by standard plate count. An inoculum of 3×10^8 CFU in 2.0 ml of peptone water was prepared.

Experimental design. At 7 days of age, five pups (group A) were orally challenged with 3×10^8 CFU of *H. pylori* in 2.0 ml of peptone water. Group B (two pups) served as controls and were given peptone water alone. Group B was initially housed separately from their infected littermates. On day 7 p.i., two pups from group A and one from group B were gavaged, and the stomach contents were cultured for reisolation of *H. pylori*. In order to ascertain whether contact infection was possible, the dogs in group B were subsequently housed with their infected littermates beginning on day 23 p.i. Blood samples were collected prior to challenge

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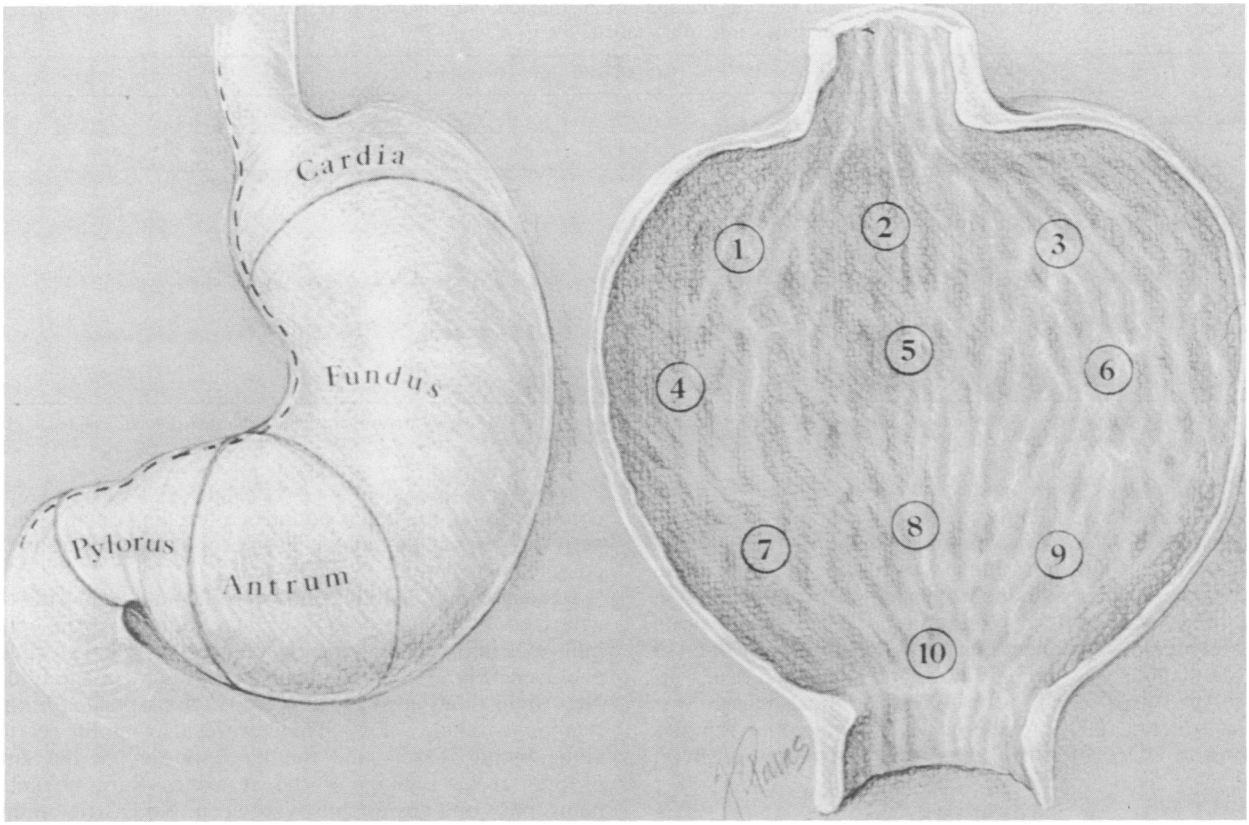

FIG. 1. Sites of biopsy for urease mapping (see Table 4).

and then once weekly until the conclusion of the study. The serum was frozen at −20°C and saved for serologic study. On day 30 p.i., the pups were anesthetized with 3.5 mg of xylazine and 17.5 mg of ketamine hydrochloride, and a pH electrode was passed into the stomach to measure the gastric pH in vivo. After this, the pups were killed with an overdose of pentobarbital.

Mucosal samples were obtained from the entire gastrointestinal tract, including the pharynx, esophagus, stomach (cardia, antrum, fundus, and pylorus), duodenum, jejunum, ileum, colon, and rectum. The gastrointestinal tract was examined for gross lesions, and samples for histopathologic examination were taken from the same regions as for culture. Multiple punch biopsies were taken from the stomach for urease mapping. Scrapings of the gastric mucosa were examined for organisms by phase-contrast microscopy.

Urease mapping. Urease mapping was performed to determine the distribution and to estimate the density of colonization by *H. pylori* in the stomach (12). Three punch biopsies each were taken from the cardia, fundus, and antrum of the stomach; one sample was taken from the pylorus (Fig. 1). The biopsy samples were incubated for 24 h in sealed microtiter plate wells containing urea, phenol red, and sodium azide in sodium phosphate buffer, pH 6.5. A positive test was detected by color change (from orange to dark pink) in the medium, and time until the change occurred was recorded. The time to positivity of this test has been shown to be proportional to the number of bacteria present at the biopsy site (12).

Microbiology. Samples taken of stomach contents by gavage on day 7 p.i. and musocal samples taken from the gastrointestinal tract at the completion of the study were streaked on blood agar (BBL Microbiology Systems, Cockeysville, Md.) and incubated for 5 to 7 days in a 10% CO_2 atmosphere. Swab samples of the isolator surfaces and samples of the food were similarly cultured. Plates were examined for growth after 7 days, and density of infection was estimated by counting the number of colonies per plate (estimated colony counts).

Histopathology. Samples taken for histologic evaluation were fixed in 10% neutral buffered Formalin and embedded in paraffin. Sections (6 μm) were stained with hematoxylin and eosin for histopathologic evaluation and with Warthin-Starry silver stain for identification and localization of the bacteria.

Serology. *H. pylori* isolated at the time of necropsy was grown for 4 days on 5% sheep blood agar in a 10% CO_2 atmosphere and used to prepare antigen for an enzyme-linked immunosorbent assay (ELISA). Bacteria were harvested in phosphate-buffered saline (PBS) containing 0.02% sodium azide (pH 7.4), washed three times, and disrupted by sonication. The preparation of antigen contained 9.5 mg of protein per ml and was stored at 4°C until use.

Antigen was diluted in 0.1 M carbonate buffer (190 μg of protein per ml, pH 9.6), and 100 μl was added to each well of flat-bottomed polystyrene ELISA plates (Immulon II; Dynatech, Chantilly, Va.). Plates were incubated at 4°C for 16 h to allow coating and then washed three times with wash buffer (PBS containing 0.05% Tween 20 and 0.1% nonfat dried milk). A blocking step was performed by incubating the wells with 200 μl of wash buffer at 37°C for 1 h, followed by aspiration of the wash buffer. Sera from the dogs were diluted in PBS containing 0.05% Tween 20 and added to each well (100 μl). Plates were incubated at 37°C for 1 h and then

TABLE 1. Microbiological findings in the four regions of the stomach and other areas of the gastrointestinal tract of gnotobiotic dogs infected with *H. pylori*[a]

Dog group and no.	Growth of *H. pylori* in culture										
	Stomach				Gastrointestinal tract						
	Cardia	Fundus	Antrum	Pylorus	Phar	Esoph	Duod	Jejun	Ileum	Colon	Rectum
Group A											
89-1021	+	+	+	+	+	+	−	+	−	−	−
89-1022	+	+	+	+	−	+	−	−	−	−	−
89-1023	+	+	+	+	+	−	−	−	−	−	−
89-1024	+	+	+	+	−	+	−	−	−	−	−
89-1025	+	+	+	+	−	+	+	−	−	−	−
Group B											
89-1026	+	+	+	+	−	−	−	−	−	−	−
89-1027	+	+	+	+	+	+	+	−	−	+	−

[a] Growth (+) or no growth (−) from mucosal tissue samples after 7 days on blood agar in a 10% CO_2 atmosphere. Phar, Pharynx; Esoph, esophagus; Duod, duodenum; Jejun, jejunum.

washed three times. Affinity-isolated alkaline phosphatase-conjugated rabbit anti-dog immunoglobulin G (IgG; Sigma Chemical Co., St. Louis, Mo.) was diluted 1:500 in wash buffer, and 100 μl was added to each well for 1 h (37°C). Wells were washed three times, and 100 μl of substrate (5-mg tablet of *p*-nitrophenyl phosphate dissolved in 10 ml of 10% diethanolamine [pH 9.6]; Sigma Chemical Co., St. Louis, Mo.) was added. After 30 min, optical density was read at 420 nm (OD_{420}) (reference, 570 nm), and samples showing an OD_{410} of ≥0.14 units were considered positive.

RESULTS

Microbiology. *H. pylori* colonized the stomachs of all group A dogs (Table 1). Culture of stomach contents obtained by gavage on day 7 p.i. resulted in recovery of *H. pylori* from the two group A dogs but not from the unexposed group B dogs. However, after 7 days of exposure to infected dogs, both group B dogs became colonized (Table 1), demonstrating that infection may be transmitted by contact. All four regions of the stomach were colonized in both groups. In addition, *H. pylori* was recovered from the pharynx, esophagus, duodenum, jejunum, and colon of some pups (Table 1). Organisms were not recovered from the isolator environment, fecal material, or food.

The degree of colonization as judged by estimated colony count was greatest in the stomach, with the fundic region tending to be the most heavily colonized (Table 2). Of the contact-exposed dogs, one was heavily colonized and one was lightly colonized in the stomach. The remainder of the gastrointestinal tract was lightly or not colonized.

Clinical signs and necropsy findings. At no time during the study did the dogs appear ill. Gastric pH was variable in the group A dogs and ranged from 1.1 to 6.4. The gastric pH of group B dogs was low (pH 1.4 and 1.9) (Table 3). In contrast to infected humans and gnotobiotic pigs, the dogs did not exhibit excess gastric mucus production. Gastric ulceration did not occur, and gross lesions, when present, were mild and consisted of multiple small (<1.0 mm) lymphoid follicles in the stomach. It was possible to demonstrate the bacterium under phase microscopy in all but one dog.

Urease mapping. Urease production was most pronounced in the fundic area (Table 4), which was also the most heavily colonized site. Unlike in humans, in whom the time until detectable urease-induced color change can be as short as 10 min, biopsy samples from the dogs required several hours for color development to occur, suggesting that bacterial colonization in the dog is not as heavy as in humans. Urease activity was not detectable in lightly colonized areas (<10 colonies per plate).

Histopathology. All dogs had chronic active gastritis characterized by focal to diffuse lymphoplasmacytic cellular infiltrates in the lamina propria. Lymphoid follicle formation occurred (Fig. 2) and was most prominent in the antrum. Neutrophils and eosinophils were often associated with the lymphoid follicles (Fig. 3) and were also lightly scattered along blood vessels and the basal border of the lamina propria of all regions of the stomach. Rare pockets of neutrophils and eosinophils were seen. No gastric epithelial erosions or ulcerations were observed. In contrast, lesions of chronic active gastritis and infiltration of the lamina propria with neutrophils or eosinophils have not been observed in uninfected age-matched gnotobiotic beagle dogs maintained under similar conditions and on a similar diet in our laboratory (M. J. Radin and S. Krakowka, unpublished data).

Inflammation was mild in the small intestine. Neutrophils and eosinophils were scattered in the lamina propria of the duodenum (four and one dog from groups A and B, respectively) and jejunum (all dogs). Follicular hyperplasia of the tonsils and ileal Peyer's patches was seen and probably represents a general response to antigenic stimulation. Both group B dogs had very mild neutrophilic-lymphocytic esophagitis. The group B dog with a positive colonic culture also had mild lymphoid follicle development in the colon.

TABLE 2. Estimated colony counts from the four regions of the stomach and gastrointestinal tract of gnotobiotic dogs infected with *H. pylori*

Dog group and no.	No. of colonies[a]				
	Cardia	Fundus	Antrum	Pylorus	GI tract
Group A					
89-1021	100–200	>500	50–100	50–100	<10
89-1022	100–200	50–100	50–100	<10	<10
89-1023	50–100	>500	>500	100–200	<10
89-1024	100–200	50–100	<10	<10	<10
89-1025	>500	>500	>500	<10	<10
Group B					
89-1026	<10	<10	<10	<10	0
89-1027	200–500	>500	>500	10–50	<10

[a] Number of colonies that grew on a plate after streaking with similar amounts of mucosal samples. GI, Gastrointestinal.

TABLE 3. Gross observations for gnotobiotic dogs infected with *H. pylori*

Dog group and no.	Lymphoid follicles[a]	Excess luminal mucus[a]	Gastric pH	Organisms[b]
Group A				
89-1021	−	−	3.3	−
89-1022	−	−	6.4	+
89-1023	1	−	1.6	+
89-1024	3	−	2.2	+
89-1025	1	−	1.1	+
Group B				
89-1026	−	−	1.9	+
89-1027	3	−	1.4	+

[a] Increasing severity of lesion from − to 3.
[b] Organisms present (+) or absent (−) in gastric mucosal scrapings observed by phase-contrast microscopy.

TABLE 4. Results of gastric urease mapping of the stomach of gnotobiotic dogs infected with *H. pylori*

Dog group and no.	Presence of urease at stomach site[a]:									
	1	2	3	4	5	6	7	8	9	10
Group A										
89-1021	+	−	−	+	+	+	+	−	+	+
89-1022	+	−	+	+	+	+	−	−	−	−
89-1023	+	−	+	+	+	+	+	−	+	−
89-1024	+	−	+	+	+	−	−	−	−	−
89-1025	+	−	+	+	+	+	−	−	−	−
Group B										
89-1026	−	−	−	−	−	−	−	−	−	−
89-1027	+	−	+	+	+	+	+	+	+	+

[a] Presence (+) or absence (−) of urease based on color change after 24 h of incubation. Sites: 1 to 3, cardia; 4 to 6, fundus; 7 to 9, antrum; 10, pylorus (see Fig. 1).

H. pylori was demonstrated in all regions of the stomach by Warthin-Starry silver staining. Bacterial colonization occurred on the surface of the stomach and appeared to be heaviest in the gastric pits. The bacteria were closely associated with the apical surface of the gastric epithelial cells, beneath the mucus layer (Fig. 4). Bacteria were not detected in Warthin-Starry-stained sections in any other area of the gastrointestinal tract.

Serology. Four group A dogs developed serum IgG specific for *H. pylori* by day 14 p.i., and all seroconverted by day 30 p.i. (Table 5). The heavily colonized group B pup had a titer of 1:200 on day 30 p.i., indicating that seroconversion may occur as early as 1 week after exposure. The second contact-exposed pup did not have detectable IgG at the end of the experiment, which may be related to the light colonization observed in this animal.

DISCUSSION

H. pylori colonizes the stomach of gnotobiotic dogs following oral challenge. The infection persists for at least 1 month after inoculation and, as in the gnotobiotic pig model

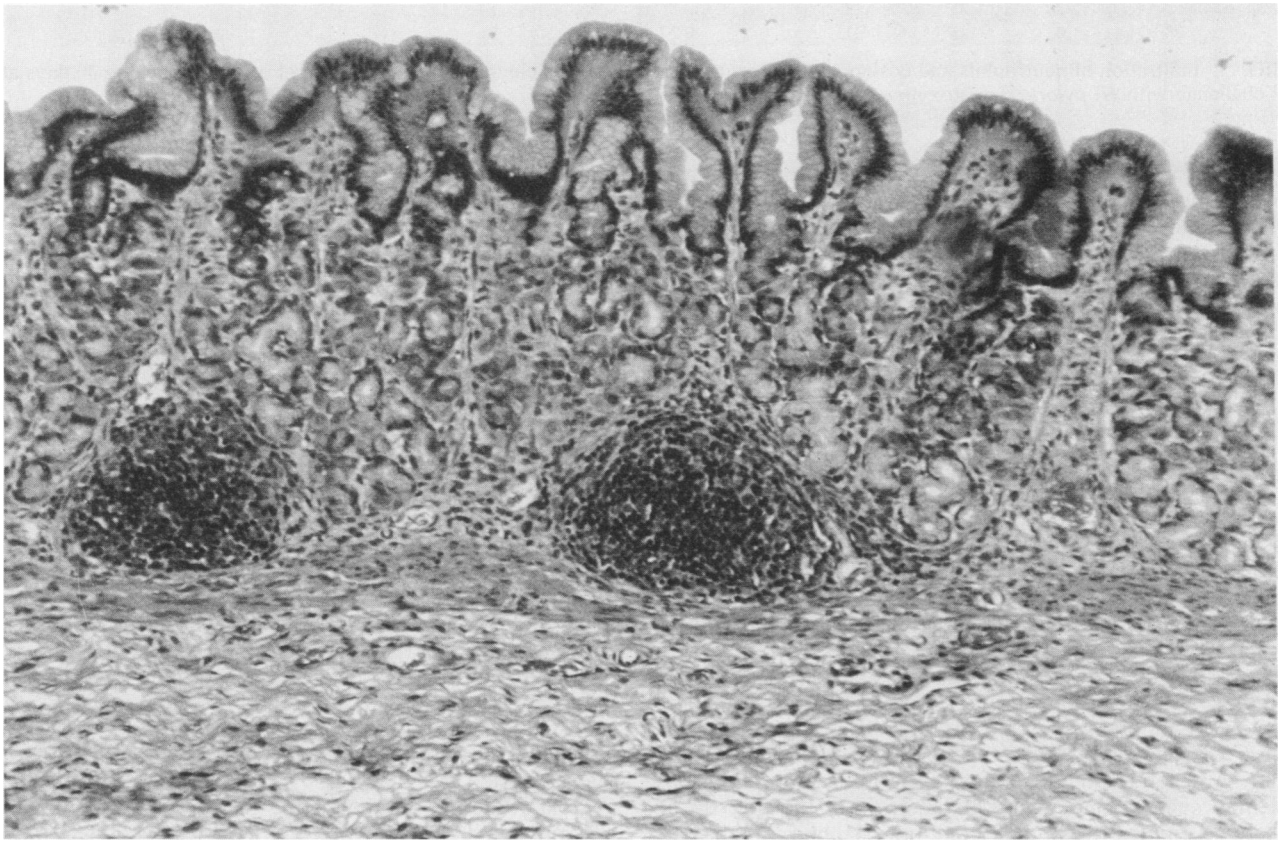

FIG. 2. Lymphoid follicle formation in the lamina propria of a gnotobiotic dog 30 days after oral challenge with *H. pylori*. Hematoxylin-eosin stain.

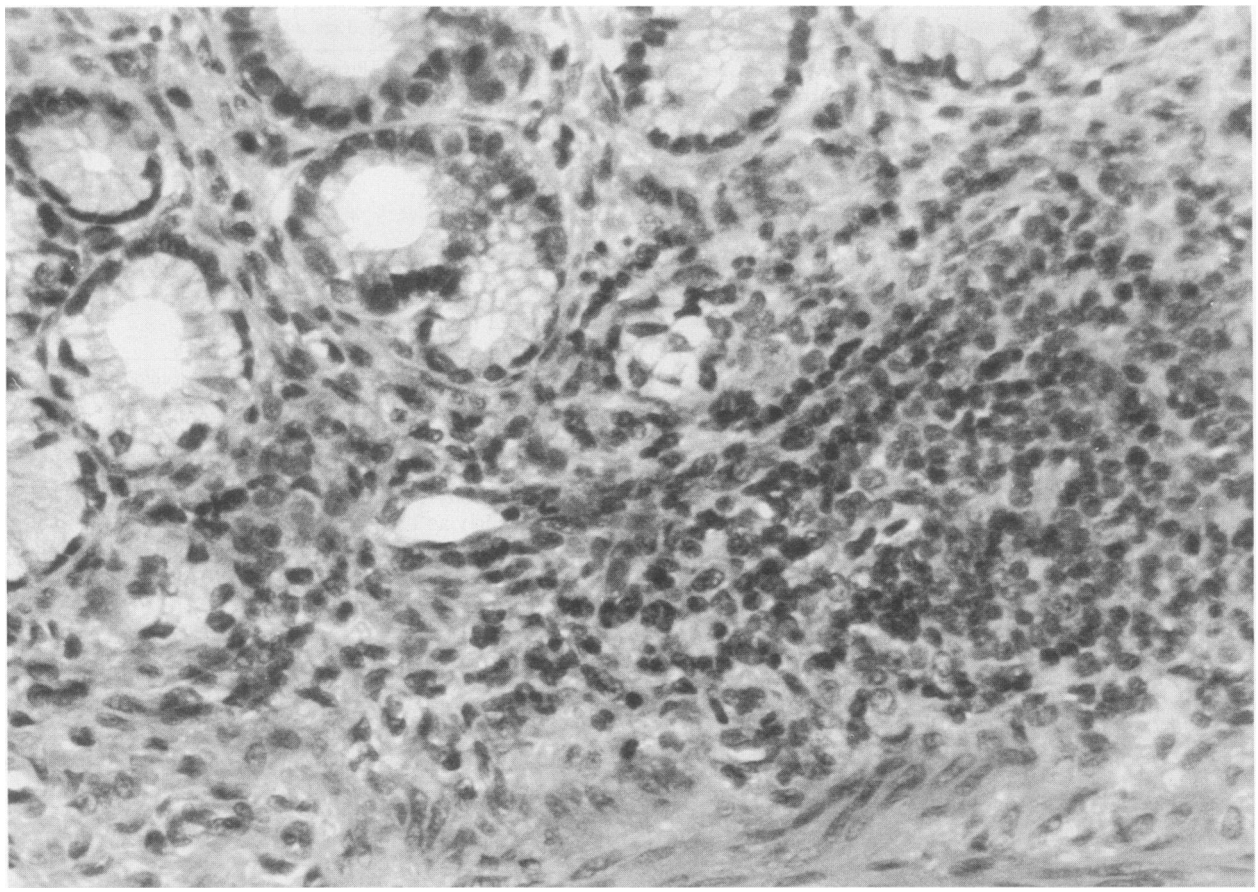

FIG. 3. Infiltration of neutrophils and eosinophils adjacent to a lymphoid follicle in the lamina propria of a gnotobiotic dog 30 days after oral challenge with *H. pylori*. Hematoxylin-eosin stain.

(15), appears to be asymptomatic. It should be noted that colonization of the gnotobiotic dog stomach as determined by urease mapping and histopathologic examination is not as marked as in human patients (12) or gnotobiotic piglets (15). In addition, the distribution of the infection is different, with the fundic mucosa being predominantly colonized in the dog versus the antrum in humans. This is consistent with the hypothesis that the human is the primary host of *H. pylori*.

In contrast to pigs and humans infected with *H. pylori*, in which colonization is restricted to the stomach, *H. pylori* was recovered by culture from other areas of the gastrointestinal tract of the gnotobiotic dog. We cannot entirely exclude the possibility that *H. pylori* reisolated from sites other than the stomach represents organisms transiently passing through the gastrointestinal tract. However, because of the presence of associated mild microscopic lesions in the esophagus, duodenum, jejunum, and colon in the dogs, the possibility of colonization of other regions of the gastrointestinal tract must be considered.

The lesions of chronic active gastritis in the dogs resembled those seen in humans (5, 18, 22). Like humans, the dogs develop a lymphoplasmacytic gastritis with continued infiltration of neutrophils. This differs from the gnotobiotic piglet, in which the neutrophilic infiltration is transient (15), and may suggest that infectious gastritis in the dog more closely resembles the human condition. Unlike in gnotobiotic pigs, gastritis in the dogs was associated with a mild eosinophilic infiltrate in the areas of inflammation. Eosinophilic infiltration has been reported in acute *H. pylori* gastritis in humans (8, 20). Eosinophilic inflammation is more often associated with parasitic infestation or allergic responses than with bacterial infection in both dogs and humans. These pups were derived from specific-pathogen-free bitches on a strict deworming program, so it is unlikely that the presence of eosinophils was the result of larval migration. An allergic response to other environmental antigens is also unlikely. Age-matched gnotobiotic pups reared under similar conditions do not have eosinophilic infiltrates or gastritis. It is probable that the presence of these inflammatory cells represents an aspect of host immune response to *H. pylori*.

Macroscopic lesions in the dogs, when present, were mild, which is similar to the case of gnotobiotic piglets and the majority of humans with *H. pylori*-associated gastritis. Ulceration was not observed. The pathogenesis of gastroduodenal ulceration in humans infected with *H. pylori* is probably multifactorial (10). It is likely that interaction of other environmental promoting agents, such as nonsteroidal anti-inflammatory drugs, smoking, and alcohol, with *H. pylori* gastritis may be required for ulcerogenesis. The gnotobiotic dog model should provide a system by which these factors may be tested.

Most of the pups had a fasting gastric pH of less than 3.0; one pup had a gastric pH of 6.4. By 5 weeks of age, normal beagle dogs tend to maintain a resting gastric pH of 3 or less and are capable of responding to histamine and pentagastrin

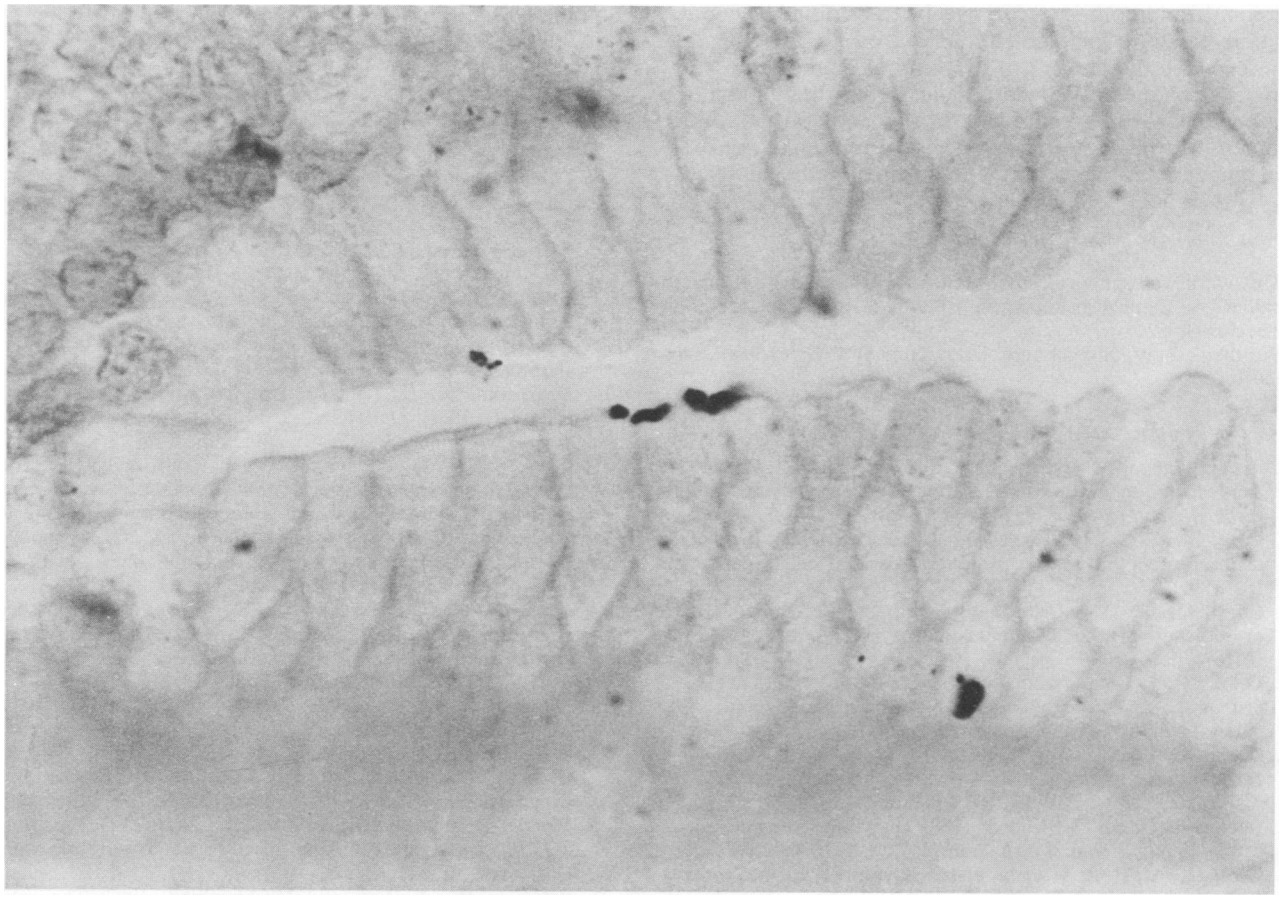

FIG. 4. Warthin-Starry stain showing the location of *H. pylori* on the surface of the gastric epithelial cells in a gastric pit of a contact-exposed gnotobiotic dog.

(17). *H. pylori* infection has been associated with transient hypochlorhydria in humans (11, 20, 21). Neutralization of gastric acid or inhibition of acid production by the bacteria may be an integral part of the disease syndrome. Recent studies suggest that *H. pylori* is capable of inhibiting acid secretion of parietal cells in vitro and that this inhibition may require attachment of *H. pylori* to gastric epithelial cells (2, 4). Studies with humans have shown that *H. pylori* may exist in the stomach under a variety of gastric acid secretory states, and the time sequence and importance of hypochlorhydria is unknown (1). In the gnotobiotic dogs, the gastric pH measured 1 or 3 weeks after challenge was not correlated to either severity of histologic lesions or number of bacteria recovered.

TABLE 5. Serum IgG antibody response to *H. pylori* over time as measured by ELISA

Dog group and no.	IgG (ELISA titer) on day p.i.:			
	0	14	21	30
Group A				
89-1021	—[a]	—	1:50	1:50
89-1022	—	1:200	1:100	≥1:400
89-1023	—	1:25	—	1:25
89-1024	—	1:50	1:50	1:200
89-1025	—	1:50	1:20	≥1:400
Group B				
89-1026	—	—	—	—
89-1027	—	—	—	1:200

[a] —, Not detectable.

It is unknown how *H. pylori* is transmitted or what the source is for reinfection of treated patients. Our data show that *H. pylori* is transmissible by contact from infected to uninfected dogs. The mechanism by which this transmission occurs was not determined and may be either oral-oral or fecal-oral. The presence of mild esophagitis in the two contact-exposed dogs suggests that colonization rostral to the stomach plays a role in early infection and transmission of *H. pylori*. In a recent survey, *H. pylori* was isolated from dental plaque of one human patient with concurrent gastritis. (13). Further work is needed in this area.

Most of the group A pups had detectable specific IgG titers for *H. pylori* by 2 weeks postchallenge. Seroconversion may take place as early as 1 week after exposure, as seen in the one contact-exposed dog, and may be related to the severity or duration of colonization. This rapid rise in serum IgG is in contrast to that seen in humans, in whom 3 or more weeks may be required to produce a detectable IgG response (8, 20). As in humans, rising IgG titers in dogs were not accompanied by clearing of the infection.

In conclusion, *H. pylori* will persistently colonize the gastric mucosa of gnotobiotic dogs for at least 1 month. The resultant disease syndrome resembles the human condition, with the production of chronic active gastritis and serocon-

version. In addition, *H. pylori* may be transmitted by contact from infected to uninfected dogs. Despite the apparent differences in degree and distribution of colonization compared with humans, our data indicate that the gnotobiotic dog may provide a good model for the study of therapeutic regimens as well as strategies for the prevention of transmission and early colonization by this human pathogen.

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