

A Euthymic Hairless Mouse Model of *Helicobacter pylori* Colonization and Adherence to Gastric Epithelial Cells In Vivo

NOBUTAKE KIMURA,¹ MASATO ARIGA,¹ FAUSTINO C. ICATLO, JR.,^{2*} MASAHIKO KUROKI,²
MOTOYASU OHSUGI,² YUTAKA IKEMORI,² KOUJI UMEDA,²
AND YOSHIKATSU KODAMA²

*Fine Chemicals Research Laboratory, Nisshin Flour Milling Co., Ltd., 5-3-1 Oi-machi, Iruma-gun,
Saitama 356,¹ and Immunology Research Institute, Ghen Corporation,
839-1 Sano, Gifu City, Gifu 501-11,² Japan*

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The hairless mouse strain NS:Hr/ICR was examined as a potential small animal model of *Helicobacter pylori* colonization, adherence to gastric epithelial cells in vivo, and gastritis. Among several small animals tested, NS:Hr/ICR mice proved to be the most highly susceptible to *H. pylori* infection. Challenge with clinical isolates of *H. pylori* consisting of either phenotype I or II (VacA and CagA positive and negative, respectively) resulted in colonization by mucus-resident and epithelial cell-adherent bacterial populations. Cell-adherent bacteria resisted 80 cycles of top-speed vortex washing and were recovered only by homogenization of serially washed glandular stomach tissue, indicating intimate association with the mucosal surface. Immunoperoxidase staining of paraffin sections of gastric tissue from infected mice revealed *H. pylori* antigens localized in the glandular region of the mucosa, with some colonized areas seen in the vicinity of submucosal mononuclear cell infiltration. The latter inflammatory reaction was observed as a function of the *H. pylori* phenotype (only type I induced inflammation) and the challenge dose (only those mice challenged with 10⁸ CFU or higher showed the reaction). The NS:Hr/ICR strain of mice is a suitable miniature model of *H. pylori* infection and may prove useful in the quest for an efficacious mode of treatment for this common infection in humans.

Helicobacter pylori was first cultured in Western Australia in 1983. This gram-negative spiral bacterium has since been shown to be a common inhabitant of the human gastric mucosa and areas of gastric metaplasia in the duodenum (11, 22, 24, 31). In developing countries, more than 80% of the population is infected by age 20, whereas up to about 50% of people 50 years of age and above are infected in developed countries (10). The organism is now a recognized etiologic agent of gastritis (20–22) and has been suggested to play a role in the pathogenesis of gastroduodenal ulcers and gastric carcinoma (1, 9, 27). In particular, the vacuolating cytotoxin (VacA) and cytotoxin-associated antigen (CagA) produced by type I but not by type II *H. pylori* have been causally associated with the development of severe forms of gastroduodenal disease (4, 29). While the function of the CagA protein is unknown, epithelial vacuolation caused by VacA may produce tissue damage and ulceration. Up to this time, the search for an effective cure for gastroduodenal disease induced by *H. pylori* has been hampered by the difficulty of reproducing infection, with its accompanying pathogenic sequelae, in easy-to-manage animal models. Since a small animal is generally preferred as a model for human disease, there have been attempts to infect germ-free mice and rats (2, 5), but without much success, possibly due to host specificity restriction of the human-derived *H. pylori*. To overcome the species specificity problem, *Helicobacter* species, such as *Helicobacter felis* and *Helicobacter mustelae*, were used to generate mouse (17) and ferret (8) infection models, respectively. However, the usefulness of these species is limited, since they do not express VacA and other virulence factors required for the induction of gastric pathology, such as ulcers and in-

flammation (29). Although successful colonization has been achieved with germ-free (14) or athymic nude mice (30), these models are relatively difficult to handle compared to conventional mice. The search for a suitable animal model was extended to other species, such as gnotobiotic piglets (6, 15, 16), gnotobiotic dogs (25), Mongolian gerbils (32), and monkeys (7). It is obvious that handling these animals in large numbers is rather difficult. Recently, successful colonization and induction of gastritis was achieved in specific-pathogen-free (SPF) and conventional BALB/c and CD-1 mice (19) by using fresh clinical *H. pylori* strains for challenge. *H. pylori* CFU were recovered from gastric mucus, suggesting that mucus was a favored niche for this organism. In the present study, we describe a new strain of mouse as a model of *H. pylori* infection and extend the previous observation on mouse mucus colonization by demonstrating that *H. pylori* adheres strongly to gastric epithelial cells in vivo. Such bacterial adherence is persistent and may give rise to gastritis, depending on the challenge dose and the *H. pylori* phenotype.

MATERIALS AND METHODS

Animals. For routine challenge purposes, we used 7- to 8-week-old mice from a hairless but euthymic SPF strain (NS:Hr/ICR) from the animal breeding facility of Nisshin Flour Milling Co., Ltd., with genetic stocks deposited at the Imamichi Institute for Animal Reproduction (deposit no. IAR-NHI-9701), Toxicology Research Center, Ibaraki, Japan. The NS:Hr/ICR strain of mice is immunocompetent and not deficient in thymus. The thymus constitutes 0.12 to 0.21% of total body weight at 21 to 23 weeks of age (unpublished data). For comparative experimental infection purposes, the following animals were used: SPF Mongolian gerbils (MON/Jms/Gbs; Nippon SLC, Hamamatsu-shi, Shizuoka-ken, Japan), commercial hairless mice (HOS:HR-1; Hoshino Animal Breeder, Yashio-shi, Saitama-ken, Japan) and SPF BALB/c mice. All animals were housed in a lightweight stainless steel autoclavable pentagonal isolator (13) and given commercial food pellets and water ad libitum. The animal facility maintained a 12-h light-dark schedule.

Bacterial strains and cultivation conditions. *H. pylori* NSP335, NSP305, and NSP355 were isolated from gastric biopsy samples of patients with gastritis. NSP335 and NSP305 belonged to the type I phenotype (VacA and CagA posi-

* Corresponding author. Mailing address: Immunology Research Institute, Ghen Corporation, 839-1 Sano, Gifu City, Gifu 501-11 Japan. Phone: (058) 235-7303. Fax: (058) 235-7505.

TABLE 1. Frequency of colonization at 1 and 2 weeks postchallenge^a

Animal	Strain	No. colonized (n = 7/group)	
		Week 1	Week 2
Hairless mouse	NS:Hr/ICR	7	7
	HOS:HR-1	3	1
Mouse	BALB/c	2	1
Mongolian gerbil	MON/Jms/Gbs	0	0

^a Animals were challenged twice with 10^7 CFU of *H. pylori* NSP335 (type I). NS:Hr/ICR hairless mice and BALB/c mice were challenged at 7 weeks of age, while HOS:HR-1 hairless mice and Mongolian gerbils were challenged at 6 weeks of age.

tive) while NSP355 was type II (negative for the two antigens). NSP333 and NSP394 were laboratory-passaged strains (types II and I, respectively). The clinical strains were isolated from gastric biopsies by inoculation of Hp selective medium (Eiken Co., Ltd., Tokyo, Japan) and incubation under microaerobic conditions with the use of activated CampyPak Plus (BBL Microbiology Systems). The organism was identified by Gram staining; production of oxidase, catalase, urease, and H₂S; and reduction of nitrate. For challenge, the strains were grown in brain heart infusion broth containing 10% horse serum (Koujin-baiyo Co., Ltd., Saitama, Japan) under microaerobic conditions for 72 h in a temperature-controlled shaker.

Challenge conditions and experimental conditions for establishment of an animal model of gastric colonization. Prior to challenge, all test animals were left without food for two consecutive days. On challenge days 1 and 2, the mice were administered 10^7 CFU orally in a single dose. After challenge, the mice were given food and water ad libitum. At specific time points after challenge, the mice were bled for serological testing by enzyme-linked immunosorbent assay (ELISA) and sacrificed for bacteriological isolation and histopathological examination. The ages and number of animals, the *H. pylori* strains used, the inoculum dose, and necropsy time postchallenge are indicated in Tables 1 to 3. The experiment was divided into three phases: (i) animal model selection (Table 1), (ii) *H. pylori* strain selection (Table 2), and (iii) high-challenge dosing for gastritis induction (Table 3). Since a positive finding should yield a bacterial count of at least 10 CFU per g of gastric tissue or 10 CFU per g of gastric mucus, these were taken as the minimum detection limits for our test.

Enumeration of *H. pylori* from gastric epithelial and mucus washings. During necropsy, half of the glandular region of the stomach was obtained under aseptic conditions. The excised gastric tissue was weighed and placed in 9 ml of phosphate-buffered saline (PBS), pH 7.0, containing 0.05% cysteine HCl and 0.1% agar. To confirm and quantify gastric colonization based on gastric epithelial cell adhesion by CFU of *H. pylori*, the gastric tissue section was washed by vortexing it 10 times at top speed in the above-mentioned PBS medium (about 2 s each time). This was done in a total of eight consecutive tubes for a total of 80 cycles of vortexing. Finally, the serially washed gastric tissue was dabbed with sterile filter paper to absorb water and the weight of the mucus-free tissue was taken. The tissue was then homogenized with a glass homogenizer in 9 ml of PBS medium. About 0.1 ml obtained at each washing step and from the supernatant of the gastric homogenate was inoculated on Hp selective agar plates and incubated for 5 days at 37°C under microaerobic conditions. CFU of *H. pylori* were identified according to the procedures described in "Bacterial strains and cultivation conditions." The presence of *H. pylori* CFU in the mucus wash or gastric tissue homogenate was considered indicative of colonization.

Histopathology. The other half of the glandular region of the stomach remaining after bacteriological testing was fixed in 10% buffered formalin and processed for histopathological examination by standard methods for hematoxylin-eosin (HE)-stained sections.

Antigen preparation for immunoassay plates and antibody titration by ELISA. Antigen was prepared from the NSP335 strain of *H. pylori* by a procedure previously described (18) for *Helicobacter hepaticus* ELISA. For coating micro-wells, the antigen was diluted to 5 µg/ml in carbonate buffer, pH 9.6, and 100 µl was dispensed into each well. After overnight incubation at 4°C, the plates were washed three times with PBS-Tween 20 (0.05% Tween 20) and blocked with 3% bovine serum albumin (fraction V; Miles) for 1 h at 37°C. The plates were washed again three times with the same wash buffer, and 100 µl of the test mouse serum prediluted 1:100 was dispensed into each well. After 1 h at 37°C, the wells were washed three times and 100 µl of horseradish peroxidase-anti-mouse immunoglobulin G (IgG) conjugate in suitable dilution was dispensed into each well. After 1 h at 37°C, the plates were washed five times and 100 µl of substrate (*ortho*-phenylenediamine) was dispensed into each well. After 10 to 15 min of incubation at room temperature, the reaction was stopped with 50 µl of 3 N H₂SO₄/well. Absorbance was read at 490 nm. Two wells per plate, not coated with antigen but treated similarly to the test wells, were used as the controls, and their optical density (OD) was subtracted from all test well readings. An absor-

bance of twice, or more than twice, that of the negative control was taken as positive. Differences in mean OD between groups were tested for significance by the Student *t* test.

Immunoperoxidase staining of paraffin sections. Paraffin sections of mouse stomach tissue were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol. For immunostaining, a Histofine kit (Nichirei, Tokyo, Japan) was used. All steps were performed at room temperature according to the manufacturer's instructions. Briefly, after a 5-min wash in PBS, the slides were blocked sequentially with 3% hydrogen peroxide in methanol and goat serum. The primary antibody consisted of a 1:4,000 dilution of rabbit anti-whole-cell *H. pylori*, while the second antibody was biotinylated goat anti-rabbit IgG. The horseradish peroxidase-streptavidin conjugate was allowed to bind with the biotinylated rabbit IgG, and diaminobenzidine (Histofine kit; Nichirei) was used as the substrate to detect the enzyme.

RESULTS AND DISCUSSION

The susceptibility of different candidate animal models to *H. pylori* colonization was examined as a first step, using a type I phenotype expressing both CagA and VacA antigens as a challenge strain. Table 1 shows that strain NS:Hr/ICR of hairless mice showed the highest degree of susceptibility to colonization by *H. pylori*. All test mice of this strain were colonized by 1 and 2 weeks postchallenge, while other test strains of mice had lower frequencies of positive gastric bacterial isolation, which further declined at 2 weeks postchallenge. The strain of Mongolian gerbils tested was negative at both 1 and 2 weeks of testing.

The difficulty in infecting SPF or conventional mice with *H. pylori* for use as an animal model of gastritis that mimics the human disease could be overcome by using a method recently described by Marchetti et al. (19). In that study, SPF or conventional BALB/c or CD-1 mice were deprived of food for 24 h before being challenged with fresh clinical isolates of *H. pylori* at days 0, 3, and 5. In the present study, we used a hairless but euthymic SPF mouse strain and a slightly different challenge protocol to produce results similar to those obtained by the above group. To colonize hairless mice, we routinely deprived the mice of food for 2 days and challenged them twice, on consecutive days, using fresh clinical isolates of *H. pylori*. The two-day fast was expected to bring down the number of antagonistic microflora, specifically *Lactobacillus* spp. (28). Our own unpublished observations showed that in mice, an initial *Lactobacillus* titer of 4.2×10^7 CFU/100 mg of gastric tissue was reduced to 2.0×10^7 CFU after 1 day of fasting and to 7.2×10^6 CFU after the second day of fasting. Moreover, the population level of the *Lactobacillus* spp. that usually predominate in the stomachs of mice and are attached to epithelial cells (12, 26) may determine the outcome of infection with certain gastric pathogens, including *H. pylori*. An inverse relationship was found between the population levels of *H. pylori* and *Lactobacillus* spp. (12) in the mouse stomach. These data support the logic behind fasting before challenge, as was done in this study,

TABLE 2. Frequency of colonization among NS:Hr/ICR hairless mice challenged with type I or II strains of *H. pylori*^a

Strain	Pheno-type	Dose (1st/2nd) (CFU)	No. colonized (n = 5/group)			
			Week 1	Week 2	Week 3	Week 4
Control		Noninfected	0	0	0	0
NSP394	I	$2.4 \times 10^7/2.8 \times 10^7$	1	0	0	0
NSP333	II	$3.3 \times 10^7/3.4 \times 10^7$	2	0	0	0
NSP335	I	$1.8 \times 10^7/2.1 \times 10^7$	5	5	5	5
NSP305	I	$1.7 \times 10^7/1.9 \times 10^7$	5	5	5	5
NSP355	II	$1.4 \times 10^7/2.1 \times 10^7$	5	5	5	5

^a Mice were challenged with 10^7 CFU at 8 weeks of age and examined at weeks 1, 2, 3, and 4 postchallenge.

TABLE 3. Quantitative recovery of *H. pylori* NSP335 and NSP355^a

Colonizing strain (phenotype)	Wk	Log ₁₀ CFU geometric mean titer of <i>H. pylori</i> in:									No. colonized (n = 5)	No. gastritis positive (n = 5) ^c
		Gastric mucus from wash step:								Gastric tissue ^b		
		1	2	3	4	5	6	7	8			
NSP335 (I)	2	6.2	ND	5.5	ND ^d	5.3	ND	ND	5.1	5.0	5	0
	4	5.8	ND	4.9	ND	4.6	ND	ND	4.2	4.3	5	0
	8	5.4	ND	5.4	ND	4.8	ND	ND	4.8	4.8	5	3
NSP355 (II)	2	5.8	ND	5.1	ND	4.9	ND	ND	5.1	5.4	5	0
	4	6.3	ND	5.3	ND	5.3	ND	ND	5.5	5.7	5	0
	8	6.7	ND	6.0	ND	5.9	ND	ND	4.8	6.4	5	0

^a Bacteria were recovered from gastric mucus and epithelial cell washings at 2, 4, and 8 weeks after challenge of 10-week-old NS:Hr/ICR hairless mice with concentrated *H. pylori* cells. The challenge doses of *H. pylori* used were 4.0×10^8 and 1.0×10^9 CFU for NSP335 and 2.6×10^9 and 3.0×10^9 CFU for NSP355. The corresponding frequency of colonization and positive histopathological findings are indicated for each examination conducted at weeks 2, 4, and 8 postchallenge. Gastric tissues from all control nonchallenged mice were negative for *H. pylori* CFU at the second, fourth, and eighth weeks of observation ($n = 5$ for each necropsy time).

^b CFU per 100 mg of homogenate.

^c By histopathology.

^d ND, not determined.

in order to bring down the number of lactobacilli that may inhibit *H. pylori* colonization.

In our experience, the population of lactobacilli in NS:Hr/ICR hairless mice was at least 50 to 100 times lower than that in conventional BALB/c mice. This may also have contributed to the higher susceptibility of this mouse strain to *H. pylori*. Thus, seven of seven NS:Hr/ICR mice were infected at weeks 1 and 2 postchallenge with 10^7 CFU of type I *H. pylori*, compared to two of seven and one of seven BALB/c mice infected at the same two time points, respectively (Table 1). The infection rates in BALB/c mice at weeks 1 and 2 in the present study were slightly lower than those in an earlier study, where two of four and four of four were infected at weeks 1 and 2, respectively (19). The difference may be related to the number of challenges performed (two in this study and three in the earlier study) and/or the bacterial strain and dose used (10^7 CFU in this study and 10^9 CFU in the earlier study). However, we cannot rule out other, yet-unknown genetic properties of NS:Hr/ICR that may account for the relatively high frequency of infection in this mouse strain compared to those in BALB/c or other strains or species tested.

Using the mouse strain NS:Hr/ICR as a colonization model, we examined the ability of different *H. pylori* strains to colonize the gastric mucosa. The results (Table 2) showed that the laboratory-passaged strains, NSP394 and NSP333, failed to establish gastric infection beyond 1 week postchallenge. All fresh clinical isolates consistently colonized the gastric mucosa, regardless of phenotype. This infection pattern was observed up to the fourth week postchallenge. However, we failed to observe evidence of gastric pathology at 4 and 8 weeks (data not shown) of infection among HE-stained sections of gastric tissue from mice infected with about 10^7 CFU. We decided, therefore, to increase the challenge dose by centrifuging bacterial cells in brain heart infusion broth culture and using the resulting cell pellet for challenge. This gave a 10- to 100-times-more-concentrated bacterial load for oral dosing. With this inoculum, and with type I or type II clinical isolates of *H. pylori* as the challenge strain, the NS:Hr/ICR hairless mice were CFU positive from week 2 through week 8 postchallenge (Table 3). A *H. pylori* count conducted on these mice during week 8 yielded relatively high titers for both the gastric mucus and washed gastric epithelial cell homogenates. The titers recovered were 10^{4-8} CFU/100 mg of gastric homogenate for the NSP335 strain (phenotype I) and 10^{6-4} CFU/100 mg of gastric homogenate for the NSP355 strain (phenotype II). These titers

were significantly different from those obtained with 10^7 CFU as the challenge inoculum (zero count at 8 weeks postinfection). Clinically, culture-positive mice did not show any sign of digestive disturbance until the end of the observation period. Body weights were similar for both infected mice and noninfected control mice (data not shown).

Gastric colonization of the NS:Hr/ICR mice invariably gave rise to mucus-resident and epithelium-adherent *H. pylori*. We failed to completely detach adherent cells by extensive washing of gastric tissue, indicating that the physical association was intimate, perhaps involving cytoplasmic invaginations harboring *H. pylori* cells, as had been observed in a recent study on polarized T₈₄ human intestinal cell monolayers infected in vitro with *H. pylori* (3). Such adherent forms were shown in that study to be resistant to gentamicin, and susceptibility was restored when the cells were lysed to release the embedded *H. pylori* organisms from cytoplasmic pouches. This finding may explain the remarkable tenacity with which the bacteria resisted 80 cycles of vortex washing at top speed in the present study. The close association with epithelial cells persisted for as long as 8 weeks, which was the last observation time point in this study. Despite their small number (about 2 to 4% of the total gastric population), such adherent bacterial cells have the potential to induce inflammation because of their proximity to gastric epithelial cells. It will be of interest to evaluate the in vivo susceptibility of such adherent cells to antimicrobial therapy to better explain their role in persistence or relapse of infection after treatment.

Mice given 10^8 to 10^9 CFU of *H. pylori* during challenge exposure were tested for serum antibody with whole-cell lysate of *H. pylori* as the ELISA capture antigen at 4 and 8 weeks postchallenge. There was a progressive increase of relatively low antibody titers, indicating access to the systemic circulation by *H. pylori* antigen, possibly in the form of bacterial breakdown products or secretions. At 2 weeks postchallenge, antibody was barely detectable. At this time point, mean ELISA ODs and corresponding standard deviations were 0.142 ± 0.003 ($n = 4$) for the type I-infected group and 0.180 ± 0.008 ($n = 3$) for the type II-infected group, which were almost the same as that of the control (0.115 ± 0.002 ; $n = 2$). At 8 weeks postchallenge, the mean ELISA ODs and corresponding standard deviations were 0.395 ± 0.280 ($n = 4$) for the type I-infected group and 0.213 ± 0.007 ($n = 4$) for the type II-infected group versus 0.113 ± 0.002 ($n = 2$) for the control. Although the infected groups had higher mean ODs (about twice or more) than the

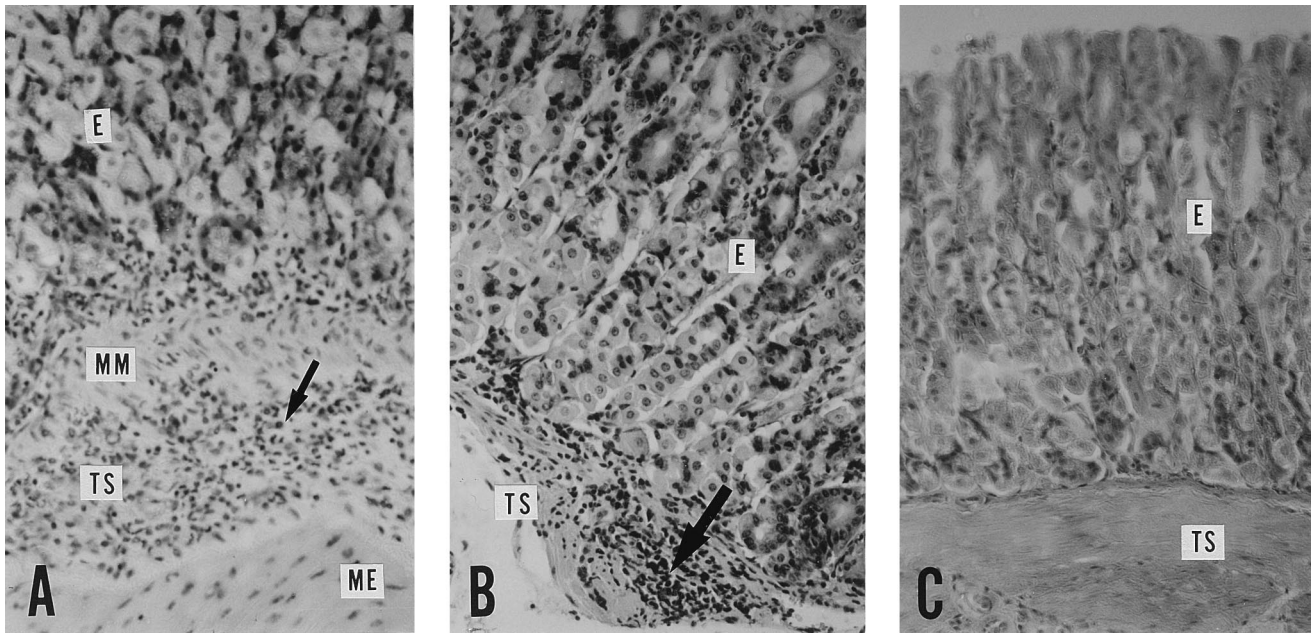


FIG. 1. (A and B) HE-stained sections of glandular stomach from hairless mice challenged 8 weeks earlier with 4×10^8 and 1×10^9 CFU of type I *H. pylori* (NSP335 strain). The tunica submucosae was infiltrated with mostly mononuclear cells (arrows). (C) HE-stained section of stomach from a hairless mouse challenged 8 weeks earlier with 2.6×10^9 and 3×10^9 CFU of type II *H. pylori* (NSP355 strain). The tunica submucosa, devoid of mononuclear cell infiltration in this mouse, was histologically indistinguishable from those of noninfected mice. TS, tunica submucosae; MM, muscularis mucosae; E, gastric epithelial cells; ME, muscularis externa (the lamina propria is not recognizable at this magnification). (Magnification, $\times 50$.)

control group, the difference was not statistically significant ($P > 0.05$).

Mice challenged with the lower dose of 10^7 CFU of either strain of *H. pylori* and necropsied at 8 weeks postchallenge did not show any histologic lesions (not shown) in the gastric mucosa. However, mild-to-severe inflammatory cell infiltration indicative of gastritis was observed histologically at the eighth week in three of five mice challenged with the higher dose of 10^8 to 10^9 CFU of type I *H. pylori* but not in the type II-challenged mice (Table 3). Histological examination at the sixth week in the group of mice given the high dose of the type I strain revealed negative results (data not shown), indicating that gastritis emerged at some time between the seventh and eighth weeks postchallenge. The negative histopathologic findings in two of five mice may be technical in origin (half of the glandular stomach was used for bacterial isolation) or may represent a late onset (after 8 weeks) of inflammation. It will be interesting to see how long infection will persist beyond 8 weeks and whether chronic *H. pylori* infection will give rise to neoplastic changes. A gastric cancer model for *H. pylori* has been elusive up to this time.

The failure to induce gastritis in mice given a challenge dose of 10^7 CFU of the type I strain and in those given a challenge dose of 10^8 to 10^9 CFU of the type II strain indicates that gastritis is a function of the bacterial load and the phenotype of the infecting strain. The absence of inflammatory cell infiltration in the gastric tissue of mice challenged with the higher dose of type II *H. pylori* also indicates a causal relationship between inflammatory changes and *VacA* and/or *CagA* phenotype, as had been observed in humans (4) and experimental animals (19). Among gastritis-positive mice challenged with the higher dose of type I strain organisms, histologic lesions consisted of mild-to-severe lymphocytic infiltration of the lamina propria and submucosal layer in the glandular region of the stomach (Fig. 1). Gross changes in the mucosa were not evident, nor were they expected, since intact gastric epithelial

lining was visualized microscopically. By immunoperoxidase staining with rabbit anti-whole-cell *H. pylori* as a primary antibody, gastritis-positive tissue sections revealed the presence of bacterial antigen, visible as dark brownish granules in the

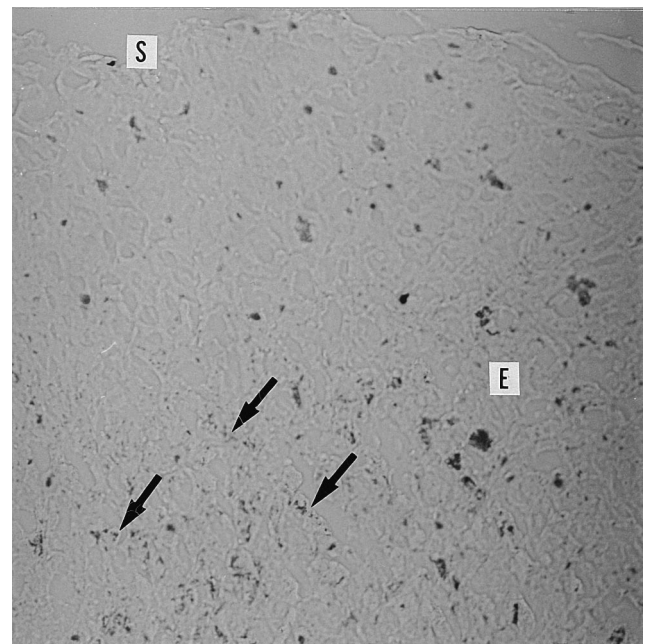


FIG. 2. Immunostained section of the glandular area of the stomach of a hairless mouse showing the region distal to the focus of mononuclear cell infiltration shown in Fig. 1A. Immunoperoxidase staining was used, with rabbit anti-whole-cell *H. pylori* as the primary antibody. The dark granular bodies (arrows) represent *H. pylori* antigen. S, gastric mucosal surface; E, gastric epithelial cells. (Magnification, $\times 50$.)

glandular isthmus, which could often be seen distal to the area of submucosal inflammatory infiltration (Fig. 2). Type II-infected mice had no observable microscopic lesions (Fig. 1C), similar to the control mice (not shown).

The natural susceptibility of a host to infection by a pathogenic organism results from interplay among the host, parasite, and environment, with the net effect of allowing the invading pathogen to occupy a niche inside the targeted host. From this favorable location, the pathogen may multiply and cause cell or organ damage and/or release other virulence factors. The present study addressed this issue in the development of a mouse model for *H. pylori* by prechallenge reduction of lactobacilli by dietary stress, selection of a susceptible strain of mice, and identification of fresh clinical isolates as colonizing strains at suitable challenge doses. The use of a convenient small animal model, such as the NS:Hr/ICR mouse, for evaluation of therapy aimed at eliminating mucus-resident and epithelium-adherent *H. pylori* cells should prove useful in the quest to eliminate this common bacterial infection from humans worldwide.

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