Experimental Infection of Mongolian Gerbils with Wild-Type and Mutant Helicobacter pylori Strains

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Experimental Helicobacter pylori infection was studied in Mongolian gerbils with fresh human isolates that carry or do not carry cagA (cagA-positive or cagA-negative, respectively), multiply passaged laboratory strains, wild-type strain G1.1, or isogenic ureA, cagA, or vacA mutants of G1.1. Animals were sacrificed 1 to 32 weeks after challenge, the stomach was removed from each animal for quantitative culture, urease test, and histologic testing, and blood was collected for antibody determinations. No colonization occurred after ≥20 in vitro passages of wild-type strain G1.1 or with the ureA mutant of G1.1. In contrast, infection occurred in animals challenged with wild-type G1.1 (99 of 101 animals) or the cagA (25 of 25) or vacA (25 of 29) mutant of G1.1. Infection with G1.1 persisted for at least 8 months. All 15 animals challenged with any of three fresh human cagA-positive isolates became infected, in contrast to only 6 (23%) of 26 animals challenged with one of four fresh human cagA-negative isolates (P < 0.001). Similar to infection in humans, H. pylori colonization of gerbils induced gastric inflammation and a systemic antibody response to H. pylori antigens. These data confirm the utility of gerbils as an animal model of H. pylori infection and indicate the importance of bacterial strain characteristics for successful infection.

Helicobacter pylori is an important bacterial pathogen that causes chronic gastritis and is associated with gastroduodenal ulcer disease, adenocarcinoma of the distal stomach, and gastric lymphoma in humans (13). Animals used for studying experimental H. pylori infection (19) include monkeys, dogs, piglets, domestic cats, and rodents (14, 15, 21, 31, 32, 35, 37, 46, 47). Several models described thus far are not optimal, since the animals cannot be handled with ease or in large numbers, germ-free conditions must be used, or infection rates are rather low. Excellent models involving Helicobacter species other than H. pylori exist (4, 20, 38), but their applicability to H. pylori infection is limited by the differences between the organisms. Infection of conventional mice with H. pylori overcomes many of these problems (40), although infection is either not easily achieved and/or is transient (31, 40). In general, the gastritis induced appears less intense than in humans (29, 40, 42, 54) or is restricted to the use of highly selected mouse-adapted strains (39), limiting the utility of these models for examining H. pylori isolates from humans.

Published results about experimental H. pylori infection in Mongolian gerbils appear promising, although somewhat conflicting. Gastritis was minimal in the initial report by Yokota et al. (59) but quite pronounced in later studies (29, 41) that used similar experimental conditions, including the same cagA-positive H. pylori strain (ATCC 43504) (29) and the same source of gerbils (29, 41). The authors (29) therefore raised the question whether the differences in histopathology could be due to the inbred gerbil strain (MGS/Sea; Seiwa Experimental Animals, Fukuoka, Japan) they used. The histomorphology of the gerbil stomach corresponds closely to that of the mouse and rat stomach (24). Naturally acquired gastritis among gerbils is rare, occurring in only 3 (2%) of 141 animals examined, in each case as a necrotizing form (6). Moreover, natural infection of gerbils with Helicobacter species does not appear to occur (5, 57). These points suggest that Mongolian gerbils are suitable to study experimental H. pylori infection. Our goals were to evaluate whether infection with wild-type and mutant H. pylori strains in Mongolian gerbils induces gastritis and to define parameters important for gastric colonization and inflammation.

MATERIALS AND METHODS

Animals and housing. Outbred Mongolian gerbils (Meriones unguiculatus) with a body weight of 30 to 50 g, corresponding to an age of approximately 4 to 8 weeks (9, 26), were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, Ind.) (Hsd:MON) or Charles River Laboratories (Wilmington, Mass.) (Crl: (MON)BR (outbred)). Up to six animals per solid-bottom cage were housed in the Animal Care Facilities of Vanderbilt University in a room with a 12-h light-dark cycle at 21 to 22°C and fed a standard commercial rodent chow. Access to food and water was free throughout all experiments. No special pretreatments (such as acid inhibition or antibiotics) were used before orogastric H. pylori inoculation or before the animals were sacrificed. Since only a few experiments were carried out with female gerbils, all other experiments were done with male gerbils when not otherwise indicated. All experiments and procedures carried out on the animals had been approved by the institutional Animal Care Committee of Vanderbilt University (protocol M95/291).

H. pylori wild-type and mutant strains used for inoculation. Fresh human cagA-positive (B127, B128, and B129) or cagA-negative (SW18, B102, B108, and B120) H. pylori isolates were obtained from patients with duodenal ulcers (strains B127, B128, and SW18) or gastritis alone (strains B128, B102, B108, and B120) who were undergoing gastrointestinal endoscopy at the Nashville Department of Veterans Affairs Medical Center or the Gastroenterology Division, University Hospital, Zurich, Switzerland. The isolates were grown on Trypticase soy agar plates with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) for 3 to 5 days under microaerobic conditions and characterized as H. pylori, as previously described (56). The cagA status was determined by PCR with chromosomal DNA, as previously described (55). Aliquots of the human H. pylori isolates with fewer than five in vitro passages were frozen, and stock cultures were maintained at ~70°C in brucella broth (BBL) supplemented with 15% glycerol. ICR mouse-passaged cagA-positive strain CPY3401 (31), originally isolated from a duodenal ulcer patient, was kindly provided by Satoru Matsu- moto and Mikio Karita; after gerbil passages and expansion of a single colony, it was designated G1.1WT (wild type). cagA-positive H. pylori strains J223,

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Isolation and characterization of \( \text{H. pylori} \) from gerbils. Each stomach specimen (approximately 2 ml) was homogenized, and 100 μl aliquots of undiluted and 10-fold-diluted homogenate (corresponding to 1/10 and 1/100 of the entire stomach, respectively) were plated on selective Trypticase soy agar plates with 5% sheep blood. After 24 to 36 h, the cells were harvested in brucella broth (BB), and the concentration was adjusted according to the OD at 550 nm, and the cells were administered to the animals immediately after harvest. The animals were oreganically inoculated three times (days 0, 2, and 4) with 0.8 to 1.0 ml of an \( \text{H. pylori} \) cell suspension of 10\(^6\) to 10\(^7\) CFU/ml in sterile brucella broth through a feeding needle. Age- and sex-matched control animals were inoculated with identical volumes of sterile brucella broth alone. Alternatively, in one experiment, \( \text{H. pylori} \) G1.1C \( + \) and G1.1 \( + \) were grown for 24 h in brucella broth supplemented with 5% fetal calf serum for subsequent challenge of 10\(^7\) CFU in 0.8 to 1.0 ml. At predetermined times, animals were anesthetized in a CO\(_2\) chamber and sacrificed by cervical dislocation. Blood was obtained by heart puncture, the forestomach was analyzed, and the glandular stomach and first part of the duodenum were removed and opened along the lesser curvature. After removal of the gross gastric contents with forceps, the stomach was inspected macroscopically and divided in half by cutting along the greater curvature. One half was fixed in a neutral buffered formalin for histologic testing; the other half was transferred to 1 ml sterile 0.1 M phosphate-buffered saline (PBS; pH 7.4) and used for culture and the rapid urease test (RUT).

The colonies were counted after 4 and 5 days, and the results are expressed as CFU/stomach. In each case, colonies were identified as \( \text{H. pylori} \) based on their resistance to the above antibiotics, characteristic morphology on plates, and positivity for the urea and oxidase activity of multiple colonies collected with a swab from the primary-culture plate. RUT was performed by incubating 100 μl of undiluted homogenate with 100 μl of filter-sterilized 0.05% phenol red–urea in 0.01 M sodium phosphate (pH 6.5) (27) at room temperature and was evaluated after 12 h. As controls, gastric homogenates of brucella broth-challenged animals were regularly included. Oxidase activity was tested by using the DrySlide oxidase test (Difco Laboratories, Detroit, Mich.). \( \text{G1.1} \) mutant strains were additionally tested for kanamycin resistance and XyIE activity, as described above. Chromosomal DNA extracted from the \( \text{H. pylori} \) challenge strain G1.1 and from isolates obtained after 1, 4, 8, 12, 16, and 24 weeks of colonization was examined by RAPD-PCR with 10-nt primers 1281, 1254, and 1247 (2).

Histopathological examination. Formalin-fixed tissue samples were processed routinely and stained with hematoxylin and eosin. For better visualization of \( \text{H. pylori} \) in the tissue sections, silver staining was performed. Briefly, after the deparaffinized tissue sections were sensitized for 5 min with 1% (wt/vol) uranyl nitrate and 1% (wt/vol) alkaline potassium tartrate in distilled H\(_2\)O, the sections were washed with distilled H\(_2\)O and stained with 10% neutral buffered formalin for 2 min, and 72°C for 2 min. Mutants were characterized by kanamycin resistance and by XyIE activity by using a 10 mM catechol solution in water, and the site of insertion in all mutants was confirmed by PCR with \( \text{aphA} \) primer, respectively (33); \( \text{G1.1WT} \) served as a control.

**Results**

Isolation of \( \text{H. pylori} \) G1.1WT and mutant strains. To assess its ability to colonize the gerbil stomach, the mouse-passaged strain CPY3401 was first passaged through gerbils. In contrast to the multiple in vitro-passaged CPY3401, gerbils could be colonized with this strain recovered from freezer stock, and the gerbil-passaged, reisolated, and clonally expanded strain then was called G1.1 for further use.

Having available a strain that reproducibly colonizes the gerbil stomach, we then created isogenic U\(^-\), C\(^-\), and V\(^-\) mutants of G1.1 to examine the effect of ablation of \( \text{ureA} \), \( \text{cagA} \), and \( \text{vacA} \), respectively, in G1.1 on gastric colonization and inflammation in Mongolian gerbils. The results of experimental infection of gerbils with G1.1WT and mutant strains are summarized in Table 1. Compared with the colonization rate of 98% with G1.1WT, ablation of \( \text{ureA} \) (G1.1U\(^-\)) completely abolished colonization whereas ablation of \( \text{vacA} \) (G1.1V\(^-\)) only slightly reduced the colonization rate (to 86%) (P < 0.024, Fisher’s exact test) (2, 3). Fisher’s exact test was performed by using Student’s \( t \) test for a comparison of means of independent samples or one-way analysis of variance. In two-tailed analyses, \( P < 0.05 \) was considered significant.

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ed-to-challenged animals, the mean levels of colonization determined by quantitative culture remained stable between $10^4$ and $10^5$ CFU/stomach for both G1.1WT and the G1.1C$^-$ or G1.1V$^+$ mutants during the observation periods (Fig. 1). Analysis by RAPD-PCR of G1.1 isolates recovered after 1, 4, 8, 12, 16, and 24 weeks of infection showed virtually identical patterns to those of the inoculation strain G1.1.

**Effect of the number of challenges on colonization.** To examine whether the number of challenges necessary to induce infection could be reduced, we compared the ratios of infected-to-challenged gerbils after one, two, or three orogastric inoculations with *H. pylori* G1.1. Whereas in one experiment all four animals in each group challenged one to three times ($n = 12$) were infected 12 weeks after challenge (a total of 12 animals), in another experiment in which five gerbils per group were challenged once with $1 \times 10^6$, $5 \times 10^6$, $1 \times 10^7$, or $5 \times 10^7$ CFU of G1.1, based on measurement of the OD$_{550}$, only two or three animals were infected 2 weeks after challenge with the three higher doses and one animal was infected after challenge with the lowest dose, as detected by culture (a total of 8 of 20 animals). Based on these findings, we concluded that in our hands, experimental infection of Mongolian gerbils with *H. pylori* in a single-challenge protocol would provide results that were too unpredictable. Therefore, in all future experiments, we used three challenges.

**Inoculation of fresh human *H. pylori* isolates.** After establishing colonization of gerbils with an *H. pylori* strain (G1.1) that had been both mouse and gerbil passaged and with mutants derived from this strain, and after finding no colonization with a multiply in vitro-passaged version of the same strain (see below), we examined whether gerbils could be infected with fresh clinical cagA-positive or cagA-negative isolates of human origin. All 15 gerbils challenged with one of three fresh human cagA-positive *H. pylori* isolates (B127, B128, and B129) became infected. Only 6 (23%) of 26 gerbils challenged with one of four fresh cagA-negative isolates (B108 [2 of 5], SW18 [2 of 5], B120 [2 of 6], B102 [0 of 10]) became infected, a significant ($P < 0.001$, Fisher’s exact test) difference from the results of challenges with fresh human cagA-positive *H. pylori* isolates.

Infection of gerbils with cagA-positive *H. pylori* B127, B128, or B129 has been documented for up to 8 weeks thus far. Parallel to G1.1WT and the G1.1V$^+$ mutant, comparison of colonization levels at up to 6 weeks and after 6 weeks of infection for *H. pylori* B127, B128, and B129 did not show a significant tendency of the mean CFU/stomach to decrease with time (data not shown), but long-term experiments with these strains are required. Next, we determined whether other in vitro-passaged strains were able to colonize gerbils or whether this phenomenon was specific for G1.1.

**Effect of in vitro passaging of G1.1 on colonization.** Since for several of the strains used in our animal studies, the exact number of in vitro passages after isolation from human gastric biopsy specimens was not documented, we studied the effect of sequential in vitro passaging of strain G1.1 on its ability to colonize the gerbil stomach. Comparison of gerbils infected with strain G1.1 that was freshly isolated from the gerbil stomach with those infected with strains obtained after an additional 10 to 35 in vitro passages on blood plates showed a progressive diminution of colonization ability, until after 20 passages, colonization was lost. The ratio of infected to challenged animals decreased from 10 of 10 animals challenged with G1.1 +0 to 7 of 10 challenged with G1.1 +10, 1 of 5 challenged with G1.1 +15 ($P = 0.004$), and 0 of 20 challenged with G1.1 +20 ($P < 0.0001$), resulting in a continuous decrease of the average CFU per challenged animal with increasing in vitro passage (Fig. 2). In contrast to the results obtained with the freshly isolated human strains, none of five animals challenged in each group with one of five multiply in vitro-passaged human cagA-positive *H. pylori* strains (JJ23, HPK5, HPK127, CPY3401, and 26695) became colonized.

**Attempts to reverse the effect of in vitro passaging on colonization ability.** Having defined for G1.1 the exact number of in vitro passages that abolishes its ability to colonize the gerbil stomach, we tested whether this in vitro-induced inability to colonize could easily be overcome. First, rechallenge with the G1.1 +15 isolate that was recovered from the one infected animal induced colonization in three of five animals (mean log$_{10}$ of 4.08 CFU/stomach), in contrast to one of five animals infected with the original G1.1 +15 strain (log$_{10}$ of 3.71 CFU [not significant]). Second, to determine whether the in vitro-induced changes were due to the growth of *H. pylori* on solid medium, gerbils were challenged three times with G1.1 +35 grown in brucella broth supplemented with 5% fetal bovine serum. However, none of five animals was found to be infected by 2 weeks after challenge, whereas all five control animals

<table>
<thead>
<tr>
<th>Bacterial genotype</th>
<th>No. of infected animals/no. challenged at time (wk) postchallenge:</th>
<th>Total no.</th>
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<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td>99/101</td>
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<tr>
<td>ureA$^+$</td>
<td>0/6 0/4 0/2</td>
<td>0/14</td>
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* $P = 0.024$, compared with wild type (Fisher’s exact test).
in vitro by chromosomal DNA from itself with an approximate G1.1 35 were equally urease positive in the natural competence of G1.1 35 transformed with DNA from G1.1 35 transformed with DNA from either G1.1A or G1.1V, we examined whether the ability to colonize could be transferred from the colonizing strain (G1.1+0) to the multiply in vitro-passaged strain (G1.1+35) by transforming growing G1.1+35 cells with chromosomal DNA from G1.1+0. In contrast to direct selection in vitro for a marker in a conventional natural transformation experiment, the G1.1+35 recipients were cultured to expand the population and then 10^{10} CFU was inoculated three times at 2-day intervals into gerbils to potentially select for colonizing transformants in vivo. Inocula of G1.1+0 transformed with chromosomal DNA extracted from G1.1+0 or G1.1+35 and inocula of G1.1+35 transformed with DNA from G1.1+35 were used as controls. In an additional control experiment, G1.1U  transformants were found for G1.1 0 and G1.1 35 at a similar frequency of 10^{-6} to 10^{-7}, indicating that the natural competence of H. pylori G1.1 remained unchanged during extended in vitro passage for more than 1 month. Cells of G1.1+0 or G1.1+35 were equally urease positive in the RUT. Similarly, cells of G1.1 after 0 to 70 in vitro passages were indistinguishable in Lewis' and Lewis' expression and in RAPD-PCR with chromosomal DNA (results not shown). None of five animals in each group became infected with strain G1.1+35 transformed with DNA from either G1.1+0 or G1.1+35, whereas all five animals in each group challenged with G1.1+0 transformed with DNA from G1.1+0 or G1.1+35 became infected. Similarly, none of the G1.1+35 isolates after passage through different mouse strains (BALB/cBy, C57BL/10, and C3H/HeJ) and freshly recovered from the mouse stomach was able to colonize the gerbils (54). Together, these data indicate that once lost, the ability of in vitro-passage G. pylori G1.1 to colonize Mongolian gerbils cannot easily be regained in vitro or even by in vivo passage through mice.

Effect of selected host factors on experimental infection of gerbils. In parallel with the gerbils normally purchased from Harlan Sprague-Dawley, 15 of 15 animals from Charles River Laboratories challenged with G1.1 were colonized with an average of 9.7 \times 10^4 CFU per stomach for up to 20 weeks, and the results indicate that both the systemic immune response and gastric histopathology were the same as described for the Harlan animals, consistent with the common origin of gerbils available in the United States (see below).

To examine whether the sex of Mongolian gerbils affects experimental infection, female gerbils (from Harlan) were challenged with cagA-positive (G1.1, B128) or cagA-negative (B108) H. pylori strains that had been previously passaged through male gerbils. In ongoing experiments, 2 of 3, 5 of 6, and 3 of 3 female gerbils in each group were still infected at 1, 2, 4, 8, 12, and 16 weeks, respectively, after challenge with G1.1 (19 of 21); 3 of 3, 2 of 2, 2 of 2, and 1 of 2 were still infected after challenge with B108 (9 of 9); and 1 of 1, 4, 6, 8, and 12 weeks after challenge with B128 (12 of 12). Together, these preliminary results indicate that the origin of the Mongolian gerbils purchased from different U.S. sources nor the sex of the animals markedly influences their susceptibility to the early stage of experimental infection with H. pylori.

**RUT.** Of 99 gerbils infected with G1.1WT, the RUT after 12 h was positive in 84 (85%), equivocally positive in 10 (10%), and negative in 5 (5%) of the animals. The mean log_{10} CFU \pm SEM/stomach determined by quantitative culture of samples from the RUT-positive animals (4.60 \pm 0.07) was higher than in the equivocally RUT-positive animals (4.31 \pm 0.14; not significant), and the RUT-negative animals (3.85 \pm 0.35; P = 0.013 by Student's t test, P = 0.021 by one-way analysis of variance). Of the 25 gerbils infected with G1.1V-, the number of infected animals with truly positive RUT was smaller (15 animals [60%]; P = 0.012 by Fisher's exact test) and the number with only equivocal RUT was larger (8 animals [32%]; P = 0.009 by Fisher's exact test) compared with those infected with G1.1WT, and the remaining 2 (8%) animals both were RUT negative. In contrast to the infection with G1.1WT, where the lack of RUT positivity was related to lighter colonization of the animals, for the G1.1V-infected animals the mean log_{10} CFU/stomach of the RUT-positive (4.70 \pm 0.07) and the RUT-equivocal animals (4.54 \pm 0.24) were similarly high. Of the 25 animals infected with G1.1C-, 23 (92%) had a positive RUT and 2 (8%) had an equivocally positive RUT. The gastric homogenates of a total of 40 control animals challenged with brucella broth alone, as well as those of 131 animals that did not become infected after challenge with either the original or the in vitro-passaged strain G1.1WT, mutant G1.1V- or G1.1U-, or various cagA-negative wild-type strains, were consistently negative, indicating the absence of urease-positive organisms in gastroduodenal homogenates of uninfected gerbils.

These results indicate that the RUT allowed rapid detection of H. pylori infection in Mongolian gerbils, with a sensitivity of approximately 90%, and that low colonization density or infection with G1.1V- was chiefly responsible for decreased sensitivity. Since the RUT was evaluated only after 12 h, a correlation between the CFU and the time elapsed until test positivity could not be made.

**Histopathologic testing.** Having established colonization of gerbils with several H. pylori wild-type and mutant strains, we asked whether colonization induced gastroduodenal inflammation. To exclude the possibility that gastric histopathologic changes were induced by the inoculation procedure itself, a total of 40 (control) animals challenged with brucella broth alone in different experiments were examined for histopathologic changes (the number of animals is given in parentheses) 0 (5 animals), 1 (2), 2 (7), 3 (3), 4 (5), 6 (6), 8 (5), 12 (1), 16 (2), 20 (1), 24 (1), or 32 (2) weeks after inoculation. None of these...
FIG. 3. Histologic findings in the gastric antrum mucosa in uninfected (A and B) or *H. pylori* G1-1-infected (C to I) Mongolian gerbils. (A and B) Neither inflammatory infiltration (A) nor silver-stainable spiral microorganisms (B) are visible in a 10-week-old uninfected Mongolian gerbil challenged three times with brucella broth alone 2 weeks before sacrifice. The arrows in panel B indicate a narrow band of amorphous material at the mucosal surface, which was present equally in *H. pylori*-infected and uninfected animals. (C) The mucosa of an animal infected for 4 weeks shows a mild polymorphonuclear infiltrate with minimal cryptitis (neutrophils in glandular lumina) and mild chronic inflammation. (D) Mucosal erosion (arrow) where the superficial mucosa is focally infiltrated by polymorphonuclear inflammatory cells in an animal infected for 6 weeks. (E) Ulcer in a gerbil infected for 8 weeks. The surface epithelium is interrupted (arrows) by the ulceration, and the wall is heavily infiltrated by inflammatory cells. The muscularis mucosae is indicated by a star. (F) Same gerbil as in panel E. Silver-stainable dark brown curved or spiral-shaped microorganisms are visible in the ulcer floor and in glandular lumina at the ulcer edges. (G) Transmural inflammation in an animal infected for 8 weeks, involving the gastric wall from the mucosa (right) to the serosa (left). The muscularis mucosae is indicated by a star. (H) Mucosal infiltration of an animal infected for 32 weeks with focal replacement of gastric glands. (I) Antral lymphoid aggregates, some with germinal centers, were found after 4 weeks of infection in animals with dense mucosal (top) inflammation, which could extend beyond the muscularis mucosae (bottom; arrows), as in this gerbil after 8 weeks of infection. Magnifications: ×150 (A), ×195 (B), ×194 (C), ×195 (D), ×59 (E), ×208 (F), ×59 (G), ×91 (H), ×65 (I). Hematoxylin and eosin stain (A, C to E, and G to I); silver stain (B and F).
animals, which covered the entire age range of animals evaluated for histopathologic changes in this study, developed gastric mucosal inflammation (Fig. 3A) or silver-stainable spiral organisms at the mucosal surface and particularly in gastric glandular lumina (Fig. 3B) 1 to 32 weeks after challenge. Two control animals had chronic mucosal inflammation (grade 1) of the duodenum. Similarly, the gastric histologic findings of 14 animals challenged with G1.1U and sacrificed after 1 to 12 weeks were normal. In both infected and uninfected animals, a band of amorphous material that was slightly silver stainable was visible on the mucosal surface and could occasionally hamper the identification of silver-stainable spiral organisms in this location. In contrast, silver-stainable spiral organisms evaluated as *H. pylori* were seen on the mucosal surface only when the underlying glands were infected.

Although the animals were not fasted before or after challenge, to further assess whether acute infection with *H. pylori* is stressful to the animals, the weight changes of the animals from 2 weeks before challenge with brucella broth or G1.1 until 6 weeks after challenge were compared. The increase in body weight of the infected and uninfected animals was similar and, in both groups, indistinguishable (data not shown) from the standard weight curve of unchallenged male gerbils obtained from the same supplier (26) or from male gerbils in the United States (9), which all are derived from the same few breeding pairs introduced into the United States in 1954 (9). Consistent with the normal weight increase, observation of the animals did not provide any evidence of distress from the inoculation procedure or infection. Consistent with these findings, no gastric macropathologic changes were evident at necropsy, except for an ulcer at the antrocorporal border in one animal after 8 weeks of infection with G1.1 (Fig. 3E).

The histopathologic changes in the gastric antrum of gerbils infected with strain G1.1 for up to 32 weeks are shown in Fig. 4. All the antral histopathologic scores increased gradually from 2 weeks after challenge. Whereas acute inflammation (Fig. 3C) scores essentially remained constant after 4 weeks, chronic inflammation scores steadily increased until 16 weeks. In addition to the predominant superficial inflammation of the antrum mucosa (Fig. 3D), deep (i.e., reaching beyond the muscularis mucosae) gastric wall inflammation (Fig. 3E, G, and I) occurred in the gerbils, which is in contrast to the *H. pylori*-induced gastric inflammation in humans. In parallel with the deep inflammation, *H. pylori* organisms were seen mainly in the glandular lumina (Fig. 3F), in contrast to their usual confinement to the surface epithelium and the gastric pits in humans (10). Although the total gastric bacterial counts for G1.1 as determined by culture did not change significantly over the first 32 weeks of infection (Fig. 1), the mucosal distribution of...
organisms, reflected by the numbers visualized by histologic testing, varied, with a peak in the gastric gland score in the first 3 to 4 weeks (Fig. 4). In contrast to uninfected gerbils, only 2 (3%) of 62 animals infected for ≥4 weeks with G1.1 did not show antral inflammatory changes, indicating the nearly universal induction of inflammation by experimental \textit{H. pylori} infection. Antral lymphoid aggregates (Fig. 3H) occurred after 6 weeks of infection and were observed in 30 (79%) of 38 animals by 12 weeks. A few large lymphoid aggregates contained germinal centers (Fig. 3I). Whereas the histologic scores for \textit{H. pylori} on the mucosal surface were unrelated to the mean CFU determined by quantitative culture, gastric gland scores of 1, 2, and 3 were associated with increasing log_{10} CFU in culture of 4.22 ± 0.24, 4.56 ± 0.11, and 4.65 ± 0.17, respectively; however, these differences were not significant because of the large variability of the CFU. In contrast to the antrum, no silver-stainable organisms or inflammatory changes were detectable in the duodenum of gerbils infected with \textit{H. pylori} G1.1 and only two animals also had gastric body inflammation (after 8 and 32 weeks of infection, including the animal with a gastric ulcer).

Gastric histopathologic changes induced by G1.1WT did not differ substantially from those induced by the G1.1C\textsuperscript{-} or G1.1V\textsuperscript{-} mutant for up to 12 weeks after challenge (Fig. 5); long-term comparative studies will show whether or not differences appear later. Also, preliminary results indicate that gastritis induced by fresh human clinical \textit{cagA}-positive or \textit{cagA}-negative \textit{H. pylori} isolates is similar to that induced by strain G1.1WT or its mutants (54).

\textbf{Serum antibody response to \textit{H. pylori}.} The serum antibody response to \textit{H. pylori} G1.1 was monitored from prechallenge to 12 weeks post-challenge in 21 animals (Fig. 6). \textit{H. pylori} infection was documented by culture and histologic testing in all animals. As detected by using goat anti-mouse IgG or IgM conjugates, both the serum IgG and IgM antibody responses to \textit{H. pylori} whole-cell sonicate varied substantially among individual animals (Fig. 6A and B). There was no correlation of the antibody responses with bacterial CFU at 12 weeks or with the scores for chronic superficial or deep gastric inflammation.
Whereas IgG antibody levels steadily rose for up to 12 weeks after challenge, IgM antibody levels in most animals showed a biphasic course with an early peak at 2 weeks and then a progressive rise until 12 weeks. Compared with the prechallenge antibody levels, levels from ≥2 weeks after challenge all were significantly ($P < 0.01$) higher for both IgG and IgM (Fig. 6C). This phenomenon allows confirmation of *H. pylori* infection after 2 weeks of challenge without the need to sacrifice the animals.

**DISCUSSION**

Our results confirm (29, 30, 41, 59, 60) that experimental infection of Mongolian gerbils with *H. pylori* is possible and indicate that stable colonization occurs for at least 32 weeks, which reproducibly induces gastric inflammation and a systemic immune response. For the first time, isogenic mutants were studied in this rodent model. The strains that can infect gerbils include several cagA-positive and cagA-negative human *H. pylori* isolates that now are available for in vivo studies; the availability of multiple strains is a prerequisite for the study of mixed infections.

Reproducible induction of gastritis in conventional mice thus far is restricted to selected and adapted *H. pylori* strains such as the Sydney strain (39), and several reports describe minimal or no infection with *H. pylori* strains that have not been preselected (29, 40, 42). Gastritis in animals infected for 4 weeks to 5 months with *H. pylori* G1.1 was significantly greater in gerbils than in several commonly used mouse strains (including C57BL/10, C57BL/6, BALB/c, C3H/HeJ, C3H/HeOuJ, and C3H/HeSnJ) (54). This finding suggests that gastritis induced in gerbils, at least in the short term, is more pronounced than in mice infected with strain G1.1. Induction of gastritis in gerbils infected with strains other than G1.1 (54) supports the view that *H. pylori* are suitable and are potentially superior to mice for studying experimental *H. pylori* gastritis, provided that host genotypic characteristics are not the focus of interest. The longer life span of gerbils than of mice (9) and the larger amount of gastric tissue available for histologic examination may represent additional advantages.

Consistent with previous reports from Japan (29, 30, 41), the *H. pylori* viable counts per gerbil stomach were around 10$^5$ CFU, gastric inflammation affected mainly or exclusively the antrum, and inflammation increased with increasing time after *H. pylori* inoculation. Both in our study and in the cited reports, gastric histopathologic findings were confined to *H. pylori*-infected animals as detected by culture, histologic testing, and RUT. In contrast, in gerbils that were not challenged or were unsuccessfully challenged, neither *H. pylori* nor other urease-positive organisms could be cultured from the gastroduodenal mucosa, no spiral organisms were found in silver stains in histologic examinations, RUT results were consistently negative, and no serum antibodies to *H. pylori* whole-cell sonicates were detected. In attempts to recover *H. pylori* from small intestinal, cecal, or distal colonic contents of *H. pylori*-infected gerbils by using selective and nonselective media, we could detect neither *H. pylori* nor other urease-positive bacteria. Culture of gastric homogenates from infected gerbils on nonselective blood agar plates on average yielded fewer colonies that were morphologically consistent with *H. pylori*, compared with the yield for the selective medium normally used (54). Although cultures on nonselective media usually were more heavily contaminated, the diminished *Helicobacter* growth is evidence against the concomitant presence of *Helicobacter* species other than *H. pylori*. In gnotobiotic gerbils carrying a defined six-member murine microflora, the gastrointestinal flora did not differ from that of conventional gerbils, except for the presence of larger numbers of gastric *Bacteroides* spp. in the former (5). Because gerbils, like other rodents, are coprophagic, their lower intestinal microflora could be of importance for the gastric microflora. However, in a survey of anaerobic and aerobic bacteria in the lower intestinal tract of Mongolian gerbils, organisms consistent with *Helicobacter* species were not identified (57). Although these studies were not carried out with the specific aim of identifying *Helicobacter* species, these data and the low prevalence of naturally occurring gastritis in gerbils reported in the literature (6) indicate that even if natural colonization of gerbils with *Helicobacter* species other than *H. pylori* does occur, its relevance for induction of gastritis is limited. Clearly, with the future use of the gerbil model of *H. pylori* infection in mind, further characterization of the gastrointestinal microflora of gerbils with special emphasis on *Helicobacter* species should be performed (22).

In contrast to studies from Japan, the gerbils in our experiments were challenged three times to reproducibly achieve colonization, the prevalence of gastric ulcers seemed to be lower, and intestinal metaplasia was not a prominent finding. Whether these differences are related to genetic host differences (43) and/or bacterial factors will have to be addressed in future experiments, but they suggest the possibility of establishing different disease models by appropriate selection of the gerbils and/or the colonizing *H. pylori* strains (30). The undefined genetic background of our outbred gerbils is a disadvantage compared to the use of genetically defined inbred mouse strains, which are superior for examining the effect of differences in host genotype, provided that the animals can be reproducibly infected with *H. pylori*. Although three orogastric challenges represent a greater effort during the experimental procedures, this protocol is well tolerated by the animals, never limited the number of animals included in different types of experiments, and, more importantly, ensured that lack of colonization reflects the characteristics of the bacterial strain challenge rather than slight technical differences in the challenge procedure. Whereas Matsumoto et al. (41) reported that two of their four animals had gastric ulcers after 16 weeks of infection, the ulcer incidence in the report by Hirayama is not indicated and ulcers occurred after 6 months of infection or more. Therefore, comparison of the ulcer incidences is difficult on the basis of the available data. Induction of intestinal metaplasia during *H. pylori* infection does not seem to be a general phenomenon. In contrast to the one positive report by Hirayama et al. (30), occurrence of intestinal metaplasia was not evident in our animals, and it is not mentioned in the report of Matsumoto et al. (41), who used animals from the same source as that used by Hirayama et al. Because gastric goblet cells normally occur in our uninfected animals without gastritis (54), it would be important to know whether the earlier diagnosis of intestinal metaplasia (30) was based mainly on the occurrence of goblet cells or on other criteria.

In vivo passage of *H. pylori* strains progressively reduces their ability to colonize the gerbil stomach, and the phenotypes necessary for colonization, although undefined at present, are not easily restored. However, animals could successfully be colonized with several isogenic mutants of strain G1.1 despite the in vitro time necessary to construct these from their wildtype parental strain. In contrast, six of seven unselected fresh human *H. pylori* isolates colonized the gerbil stomach. These results indicate that gerbils represent a suitable rodent model for in vivo comparisons of fresh human *H. pylori* isolates. Urease is essential for establishment of *H. pylori* colonization in gerbils, similar to gnotobiotic piglets (16), primates (28), nude mice (51), and ferrets (3). The exact contribution of urease is
unknown, but effects on local pH could be tested by attempting to establish infection in animals treated with H₂-blockers or proton pump inhibitors that would elevate gastric pH (18). The vacuolating cytotoxin (VacA) is not essential for colonization, confirming results obtained with gnotobiotic piglets (17), although the infection rate was slightly lower with the vacA mutant than with the wild-type strain. The results of the RUT suggest that the lower infection rate could be associated with decreased urease activity of the vacA mutant in vivo or with decreased levels of colonization. *H. pylori* VacA is heterogeneous (12), and some strains produce little protein in vitro. The role of VacA in vivo is unknown, and it is not certain that the induction of vacuoles in eukaryotic cells is relevant to its biological role in vivo. We do not know the levels of VacA expression in vivo in humans; vacuole production in vitro may be induced at doses many times those occurring in vivo, for example. Nevertheless, the presence of two copies of vacA homologs in the *H. pylori* genome (50) suggests that VacA has an important conserved function. The association between toxigenic *H. pylori* strains and ulcer disease in humans (7) and the animal data (obtained with piglets and gerbils) indicating that VacA is not necessary for short-term colonization and does not influence gastric inflammation in the first 3 months suggest to us that VacA may be involved in long-term rather than acute events. The degree to which results from experimental mouse challenge with sonicates from *H. pylori* wild-type or vacA mutant strains (23) are applicable to the induction of gastric histopathologic changes during natural infection is not known.

Colonization of gerbils with the isogenic cagA mutant indicates that cagA itself is not essential for colonization. Since many *H. pylori* strains do not possess cagA (11, 52) or the complete cag island (8), it clearly is not necessary for the establishment of colonization in humans. However, reduced colonization of gerbils with fresh wild-type cagA-negative compared with cagA-positive isolates suggests that cagA could be a marker for genes in the cag island or elsewhere that enhance colonization ability in gerbils. In vivo passage through gerbils appeared to increase the ability of cagA-negative *H. pylori* strains to colonize the gerbil stomach. Therefore, reduced colonization may be restricted to the interspecies (human-to-gerbil) transfer of cagA-negative *H. pylori* strains but may not be true for the intraspecies (gerbil-to-gerbil) transfer of these organisms. That inflammation during the first 3 months of infection induced by both the cagA- and vacA-ablated *H. pylori* mutants was similar to that from the wild-type strain also is consistent with the induction of gastritis in mice by *H. felis*, which does not express CagA or VacA (43). Our model provides the basis for studying the in vivo effect of ablation of essentially any *H. pylori* gene, provided that the gene product is not crucial for colonization.

Gerbils from different U.S. sources tolerate colonization with *H. pylori* well, with no change in their expected weight gain in captivity. In total, only one animal infected with strain G1.1 (2% of 48 animals observed for ≥8 weeks) developed a gastric ulcer. Whereas strain G1.1 was originally isolated from a patient with a duodenal ulcer (31), no duodenal ulcers were observed in the gerbils. This low rate of ulcerogenicity may be analogous to ulcerogenesis in persistently colonized humans, in contrast to the findings in gnotobiotic piglets (36). The histopathologic response to colonization also shows some notable similarities in the antrum between humans and gerbils, with both acute polymorphonuclear leukocyte and chronic lymphoplasmacytoid cell infiltration. That not only were 99 of 101 gerbils challenged with strain G1.1 infected, but also that 97% of the animals infected for ≥4 weeks had antral gastritis shows the potential of this animal model. The interindividual variation in the degree of inflammation may be related in part to the use of outbred animals. Selection and mating of animals with strong responses could lead to inbred gerbil strains with enhanced gastric inflammation during *H. pylori* infection. The gradual increase of the chronic deep inflammation during the first 24 weeks suggests that these deep lesions may persist for the entire duration of *H. pylori* infection, whereas the scores for chronic superficial inflammation reach a plateau after about 12 weeks. The deep inflammation extending beyond the muscularis mucosae is not observed in humans and may relate to the presence of *H. pylori* in the lumina of the gastric glands, in contrast to their usual absence in human gastric glands. The histopathologic changes induced by several human clinical *H. pylori* isolates are virtually identical to those induced by strain G1.1 (54).

The variability in antibody responses to *H. pylori* infection in gerbils parallels that observed in humans (45), and, similar to humans, chronic infection develops despite a host immune response. The present lack of gerbil-specific reagents represents a limitation of this rodent model of *H. pylori* infection; however, due to interspecies cross-reactions, mouse-specific antibodies can be used (25, 48, 53, 61) until gerbil-specific immune reagents are developed. The bacterial antigen preparation used for the ELISA to detect antibodies can be individualized based on the challenge *H. pylori* strain, with inclusion of many if not all of the relevant antigens to which immune responses may occur. Experimental infection with particular strains also excludes the possibility of mixed infection with several *H. pylori* strains differing in their antigenicity. The variability in antibody responses in gerbils may therefore reflect true heterogeneity of the host immune response to *H. pylori* antigens rather than lack of representativeness of the antigens used in the ELISA. Despite the marked variability in antibody responses between different hosts, and although isotype-specific detection of gerbil Igs by antibodies to mouse Ig isotypes may not be completely accurate, several clear trends emerge, as shown in Fig. 6C. The time course of the response is similar to that after experimental viral infection (34) or parenteral parasitic infection with *Dipetalonema viteae* or *Leishmania major*, where elevated antibody levels occur in serum after 1 week of infection (1, 58). The early peak in the IgM response is similar to that observed in a human volunteer who ingested *H. pylori* (44), although the secondary rise differs; long-term studies should indicate whether there is parallelism with phenomena in humans. That the early IgM peak immediately precedes the peak of the histologic score for *H. pylori* in gastric glands suggests the existence of at least a temporal relationship. The steady rise in IgG levels also parallels human responses; the decreasing numbers of bacteria observed in the gastric glands may reflect the development of an immune response that limits the bacterial population in this location. The increase in the IgG levels in serum and in the number of mononuclear cells in the lamina propria, after the numbers of neutrophils have reached a plateau, suggests that a mature immunologic response to the organism is developing.

In conclusion, specific advantages of the gerbil model outweigh its present limitations and suggest applications that are complementary to those of existing rodent models. Limitations include the lack of information about host genetics, gastric physiology, and gastrointestinal microflora and the lack of gerbil-specific immune reagents. Experimental infection of Mongolian gerbils with *H. pylori* represents a versatile model, since it offers the technical advantage of a small laboratory animal with good susceptibility to experimental *H. pylori* infection, a brisk gastric inflammatory and systemic immune response, and...
prolonged colonization. For colonization, bacterial strain characteristics appeared more important than host characteristics.

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