


Dietary Composition Influences Incidence of *Helicobacter pylori*-Induced Iron Deficiency Anemia and Gastric Ulceration

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Epidemiologic studies have provided conflicting data regarding an association between *Helicobacter pylori* infection and iron deficiency anemia (IDA) in humans. Here, a Mongolian gerbil model was used to investigate a potential role of *H. pylori* infection, as well as a possible role of diet, in *H. pylori*-associated IDA. Mongolian gerbils (either *H. pylori* infected or uninfected) received a normal diet or one of three diets associated with increased *H. pylori* virulence: high-salt, low-iron, or a combination of a high-salt and low-iron diet. In an analysis of all infected animals compared to uninfected animals (independent of diet), *H. pylori*-infected gerbils had significantly lower hemoglobin values than their uninfected counterparts at 16 weeks postinfection ($P < 0.0001$). The mean corpuscular volume (MCV) and serum ferritin values were significantly lower in *H. pylori*-infected gerbils than in uninfected gerbils, consistent with IDA. Leukocytosis and thrombocytosis were also detected in infected gerbils, indicating the presence of a systemic inflammatory response. In comparison to uninfected gerbils, *H. pylori*-infected gerbils had a higher gastric pH, a higher incidence of gastric ulcers, and a higher incidence of fecal occult blood loss. Anemia was associated with the presence of gastric ulceration but not gastric cancer. Infected gerbils consuming diets with a high salt content developed gastric ulcers significantly more frequently than gerbils consuming a normal-salt diet, and the lowest hemoglobin levels were in infected gerbils consuming a high-salt/low-iron diet. These data indicate that *H. pylori* infection can cause IDA and that the composition of the diet influences the incidence and severity of *H. pylori*-induced IDA.

Anemia affects up to 1.6 billion people worldwide, potentially resulting in fatigue, decreased productivity, increased susceptibility to infection, or death (1–5). A wide variety of infectious processes can be primary causes of anemia or can exacerbate anemia arising from noninfectious etiologies. For example, intestinal parasitic infections can cause chronic blood loss, leading to iron deficiency anemia (IDA; i.e., anemia due to iron deficiency) (3, 6). Anemia arises in patients with malaria because the parasites invade erythrocytes (2), and bacterial toxins can cause hemolysis (7). In addition, chronic infections, chronic immune activation, and cancer can cause a form of anemia known as “anemia of chronic disease” (8).

Colonization of the human stomach with the gastric bacterium *Helicobacter pylori* is another potential cause of anemia. Anemia often occurs in the setting of symptomatic *H. pylori*-associated diseases (peptic ulcer disease or gastric cancer), but it has been suggested that *H. pylori* may also be a cause of IDA in asymptomatic persons with no evidence of peptic ulcer disease or gastric cancer (9). *H. pylori* has been linked to several other hematologic diseases, including pernicious anemia (vitamin B₁₂ deficiency arising through an autoimmune-mediated process) and idiopathic thrombocytopenic purpura (ITP; a disorder characterized by a reduction in circulating platelets) (10–12).

Human epidemiologic studies examining a potential association between *H. pylori* colonization and anemia have yielded conflicting results (13–18). In one of the largest studies to date, serum ferritin and hemoglobin levels were analyzed in 2,794 Dutch adults to determine if *H. pylori* infection was associated with IDA. In men and postmenopausal women, *H. pylori* infection was linked to iron deficiency (as determined by serum ferritin levels) (13). Among premenopausal women, no such association was observed. Hemoglobin levels were not affected by *H. pylori* coloni-

zation status in any of the cohorts (13). A subsequent meta-analysis of existing studies detected an association between *H. pylori* infection and IDA, based on analyses of hemoglobin and serum ferritin levels (16). Conversely, several other studies have not detected any association between *H. pylori* and anemia (14, 15, 18).

Animal studies examining a potential link between *H. pylori* infection and anemia have also yielded conflicting results (19–22). In one study, *H. pylori*-infected male INS-GAS mice (which overexpress gastrin) exhibited reductions in both hemoglobin and serum ferritin levels compared to uninfected animals; however, the mean corpuscular volume (MCV; a measure of the average size of erythrocytes) was elevated in the infected cohort (21), a finding that differs from the reduced MCV typically observed in patients with IDA. Infection of INS-GAS mice with the related organism *H. felis* also resulted in anemia (19). A potential limitation of the mouse model for studying *H. pylori*-associated IDA is that *H. pylori* strains often undergo inactivating mutations in the *cag* pathogenicity island during the course of mouse stomach colonization

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(23, 24). In a study of Mongolian gerbils infected with a *cagA*-positive *H. pylori* strain (ATCC 43504), anemia was not detected in *H. pylori*-infected gerbils, although a trend toward a decreased MCV in infected animals was observed (22).

H. pylori is present in about half of the human population worldwide, and most of these people are asymptomatic. If *H. pylori* contributes to anemia in asymptomatic people, this bacterium could potentially have a substantial impact on the incidence and severity of anemia worldwide (25). The pathogenic mechanisms by which *H. pylori* might contribute to anemia in asymptomatic people are not well understood. Anemia could potentially occur due to blood loss from asymptomatic gastric erosions, impaired absorption of iron due to increased gastric pH, reduced vitamin B₁₂ levels due to atrophic gastritis and parietal cell loss, or anemia of chronic disease. Thus far, few studies have been designed to discriminate among these possibilities.

Although the majority of *H. pylori*-infected people are asymptomatic, the presence of *H. pylori* is a strong risk factor for peptic ulcer disease or gastric cancer. The factors that determine whether peptic ulceration or gastric cancer develops in individual humans are not completely understood, but several factors are relevant, including features of the *H. pylori* strain with which the host is infected, host genetic characteristics, and in the case of gastric cancer, host diet (26–32). Specifically, increased dietary salt intake and decreased dietary iron intake are associated with an increased risk of gastric cancer in *H. pylori*-infected humans and in experimentally infected gerbils (33–42). This is attributed at least in part to the enhanced virulence of *H. pylori* under high-salt or low-iron conditions (33, 35, 43). For example, high-salt conditions have been shown to cause alterations in *H. pylori* gene transcription or protein production *in vitro* and *in vivo*, including augmented production of CagA (33, 44–46). Similarly, low-iron conditions have been shown to cause alterations in *H. pylori* gene transcription and stimulate increased activity of the *cag* type IV secretion system (T4SS) (35, 47, 48). Since high-salt or low-iron diets have been associated with increased gastric inflammation and an increased severity of gastric disease in animal models of *H. pylori* infection (33, 35, 38, 49), we hypothesized that these dietary alterations might potentiate the development of anemia in the setting of *H. pylori* infection. In the current study, we investigate a potential link between *H. pylori* infection, diet, and anemia using the Mongolian gerbil model, and we define the mechanisms by which anemia arises in this model.

MATERIALS AND METHODS

***H. pylori* infection of Mongolian gerbils.** A single cohort of 96 male gerbils between the ages of 3 and 5 weeks (weight, up to 40 g) was obtained from Charles River Laboratories. The animals were divided into 4 groups (24 animals per group), each of which received a different diet. One group received TestDiet AIN-93M (Purina Mills), and the other groups received modified versions of AIN-93M: a low-iron diet (AIN-93M manufactured to contain no iron, compared to the 39 ppm iron in the normal chow), a high-salt diet (AIN-93M modified to contain an additional 8% sodium chloride, for a total concentration of 8.25% sodium chloride, compared to 0.25% in the normal chow), or a combination high-salt and low-iron diet (AIN-93M manufactured to contain no iron and 8.25% sodium chloride). Within each group, 16 animals were experimentally infected with *H. pylori* and 8 remained uninfected. The defined diets were fed to each gerbil cohort for 3 weeks prior to *H. pylori* infection and throughout the remainder of the experiment. Uninfected control gerbils were fed the corresponding diets for the same length of time. Gerbils were euthanized at 11

or 16 weeks after infection. The experiments were designed with the intent to euthanize 2 uninfected animals and 6 infected animals per diet at 11 weeks postinfection and the intent to euthanize 6 uninfected animals and 10 infected animals per diet at 16 weeks postinfection. The actual numbers of data points reported for individual assays vary slightly from these intended numbers due to several issues involving individual animals, including clotting of blood samples at the time of harvest, a suboptimal quality of gastric tissue collected for histologic analysis, or the unexpected death of animals prior to the 11- or 16-week time points. All experimental procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Inoculation with *H. pylori*. Gerbils were experimentally infected with *H. pylori* strain 7.13. Strain 7.13 is a CagA-positive strain with a functional *cag* type IV secretion system, and it has a mutation in *vacA* that abrogates VacA production (50, 51). The parental strain (B128) was originally isolated from a human with a gastric ulcer and was experimentally introduced into a Mongolian gerbil. An isolate from the gerbil was designated strain 7.13 (51). *H. pylori* strain 7.13 was grown at 37°C in room air supplemented with 5% CO₂ in sterile brucella broth supplemented with 10% fetal bovine serum (FBS) to mid-log phase (optical density at 600 nm, approximately 0.6). Gerbils were fasted overnight and then were infected via oral gavage with 1 × 10⁹ CFU. Gerbils were gavaged twice, on the first and third day of the experiment, each time fasting overnight prior to gastric challenge with *H. pylori* (33, 35).

Hematological and serum ferritin analysis. At the experimental endpoints, blood was collected from each gerbil by cardiac puncture and placed into tubes containing the anticoagulant EDTA. Complete blood counts (CBCs) were determined by the Translational Pathology Shared Research core at Vanderbilt University. CBC results included hemoglobin values, mean corpuscular volume measurements, white blood cell (WBC) counts, and platelet counts. Serum was collected at the experimental endpoint and stored at –80°C. Serum ferritin was analyzed using a ferritin enzyme-linked immunosorbent assay (ELISA) designed for use with mouse blood following the manufacturer's instructions (Kamiya Biomedical Co.) (35).

Gastric pH. Gastric pH was measured at the experimental endpoint by gently pressing pHHydrion pH paper (Micro Essential Laboratory) into the freshly incised glandular stomach at the antrum (33).

Bacterial density. To quantify the *H. pylori* density in the gastric tissue, a portion of the stomach collected at the time of harvest was weighed, homogenized in sterile brucella broth containing 10% FBS, and serially diluted onto *H. pylori*-selective tryptic soy agar plates containing 5% sheep blood (Hemostat Laboratories), vancomycin (20 g/ml; Sigma-Aldrich), nalidixic acid (10 g/ml; Sigma-Aldrich), bacitracin (30 g/ml; Sigma-Aldrich), and amphotericin B (2 g/ml; Sigma-Aldrich). The plates were incubated for 5 days at 37°C under microaerobic conditions (BD GasPak EZ Campy container system). After 5 days, the numbers of CFU were counted and the numbers of CFU per gram of stomach tissue were calculated (33, 35).

Histology. At the time of harvest, longitudinal strips of the glandular stomach were collected and fixed overnight in 10% neutral buffered formalin (Fisher Scientific). Following fixation, the tissue was embedded in paraffin, sectioned, and subsequently stained with hematoxylin and eosin. The slides were examined for gastric pathology by a pathologist who was blind to the identity of the specimens. The specimens were evaluated histologically for gastric inflammation, chief and parietal cells, gastric ulceration (characterized by mucosal disruption with inflammatory exudate and cell debris), dysplasia (characterized by irregular glands with budding or branching in the mucosa), and gastric adenocarcinoma (characterized by dysplastic glands penetrating through the muscularis mucosa into the submucosa). These criteria for dysplasia and adenocarcinoma are in agreement with consensus guidelines (52). Acute inflammation (polymorphonuclear neutrophils) and chronic inflammation (mononuclear leukocytes) were graded separately on a scale with scores ranging from 0 to 3 (absent, mild, moderate, and marked inflammation, respectively) in

both the antrum and corpus, for a cumulative total score of 0 to 12 (33, 35, 52, 53). The loss of parietal cells and the loss of chief cells were estimated semiquantitatively (as the percentage of cells lost) on the basis of a review of the hematoxylin- and eosin-stained sections containing the entire length of the stomach. Gastric ulceration, dysplasia, and adenocarcinoma were recorded as present or absent, but these features were not scored for severity. All gastric cancer cases were independently evaluated by a second pathologist (who was also blind to the experimental conditions), who confirmed the diagnoses.

Diagnostic criteria for IDA. To diagnose anemia in humans, the World Health Organization analyzes hemoglobin levels in a healthy reference population. A value 2 standard deviations below the mean hemoglobin value for this reference population is set as the lower limit of normal, and individuals with hemoglobin values below this level are considered anemic (5). To develop similar criteria for anemia in the gerbils, we used the uninfected gerbils on a normal diet as the reference population. The uninfected gerbils harvested at the 11-week and 16-week time points had similar mean hemoglobin levels (13.75 g/dl and 13.28 g/dl, respectively), and therefore, the results for uninfected animals from the two time points were pooled for most analyses. A value 2 standard deviations below the mean hemoglobin value for animals analyzed at the 16-week time point was established as the lower limit for normal hemoglobin scores; gerbils with hemoglobin levels lower than this cutoff were considered anemic. Microcytic anemia was diagnosed using a similar approach with MCV as the determinant. A value 2 standard deviations below the mean MCV for the normal, uninfected cohort at the 16-week time point was established as the lower limit for a normal MCV; gerbils with MCV values lower than this cutoff were considered to have microcytosis. To detect iron deficiency, serum ferritin values were analyzed. The normal, uninfected cohort of gerbils harvested at the 16-week time point was again utilized as the reference population. Due to a high level of variation in serum ferritin values, confidence intervals were used as a tool to establish lower limits for iron deficiency. The lower 95% confidence interval of the mean represented the lower limit of normal, and gerbils with serum ferritin values below this level were considered iron deficient. Any gerbil meeting the criteria for both anemia and iron deficiency was diagnosed as having iron deficiency anemia.

Fecal occult blood. Gerbil stool samples were collected at the time of harvest by squeezing the contents of the large intestine into Eppendorf tubes. To detect occult blood, a Hemocult Sensa (Beckman Coulter) assay kit was used following the manufacturer's instructions.

Statistical analysis. All data are presented as the mean \pm standard error of the mean (SEM). Statistical analyses were conducted using GraphPad Prism software package. All associations were assessed using Spearman's rank-order correlation.

RESULTS

Anemia in *H. pylori*-infected gerbils. To investigate the possible effects of *H. pylori* and diet on the development of anemia, we conducted experiments using a Mongolian gerbil model of *H. pylori* infection. Male Mongolian gerbils were maintained on one of four diets: a normal diet, a high-salt diet, a low-iron diet, or a combination of a high-salt and low-iron diet. Among gerbils receiving each diet, animals in one subgroup were experimentally infected with *H. pylori* and the animals in the other subgroup served as uninfected controls. At two experimental endpoints (11 and 16 weeks postinfection), *H. pylori*-infected gerbils and uninfected control gerbils were euthanized, blood was collected to examine hematological parameters, and gastric tissue was collected to assess changes in gastric pathology.

In an analysis in which all *H. pylori*-infected gerbils were compared to all uninfected gerbils (independent of diet), we observed significantly decreased mean hemoglobin values among infected gerbils at the later time point (16 weeks postinfection) in compar-

ison to the values among the uninfected gerbils (11.3 ± 0.3 g/dl and 13.4 ± 0.1 g/dl, respectively; $P < 0.05$) (Fig. 1A). Infected gerbils harvested at the early time point (11 weeks postinfection) also had reduced mean hemoglobin values compared to the uninfected cohort, but the difference did not reach statistical significance (Fig. 1A; see also Fig. S1A in the supplemental material). Among infected gerbils harvested at the 16-week time point, the cohort fed a combination of a high-salt and low-iron diet exhibited the lowest hemoglobin levels ($P < 0.05$) (Fig. 1B). Similar results were observed when hematocrit values were analyzed (see Fig. S1B and C).

To determine if anemia was present in individual gerbils, we used the criteria described in Materials and Methods. By these standards, 72% of all infected gerbils harvested at the later time point were anemic, whereas none of the uninfected animals were anemic (Fig. 1C). Anemia rates among groups of infected gerbils differed depending on the diet that they received. Infected gerbils on a normal diet had the lowest incidence of anemia (55%), and infected gerbils fed a combination high-salt and low-iron diet had the highest incidence (100%) ($P = 0.02$ compared to infected gerbils fed a normal diet) (Fig. 1C). There were no significant differences in *H. pylori* colonization levels among the cohorts (see Fig. S1D in the supplemental material). These data indicate that *H. pylori* causes anemia in experimentally infected gerbils and that the composition of the diet influences both the incidence and the severity of anemia.

Effect of diet on iron deficiency anemia in *H. pylori*-infected gerbils. As a first step in elucidating the type of anemia present in *H. pylori*-infected gerbils, we analyzed the erythrocyte mean corpuscular volume (MCV). The mean MCV was significantly lower among *H. pylori*-infected gerbils at the 16-week time point (53.9 ± 1.3 fl) than among infected gerbils at the earlier 11-week time point (63.0 ± 2.3 fl) ($P < 0.05$) or uninfected gerbils (62.7 ± 0.6 fl, on the basis of pooled data from both time points, $P < 0.05$) (Fig. 1D; see also Fig. S1E in the supplemental material), indicating the presence of a microcytic anemia (i.e., an anemia characterized by a reduction in the MCV, in contrast to a normocytic anemia, in which the MCV is not significantly reduced). The lowest MCV values were detected in infected gerbils fed either a low-iron diet (49.6 ± 2.0 fl) or a combination of a high-salt and low-iron diet (48.9 ± 2.9 fl) ($P < 0.05$ compared to the uninfected cohort on a normal diet or to the uninfected control groups on the same diets) (Fig. 1E).

The most likely causes of microcytic anemia in *H. pylori*-infected gerbils are iron deficiency anemia and anemia of chronic disease (anemia due to persistent infection or disease) (54). A distinguishing feature of IDA is a low serum ferritin concentration, whereas anemia of chronic disease is characterized by an elevated serum ferritin concentration (55). Therefore, we analyzed serum ferritin levels by enzyme-linked immunosorbent assay (ELISA). Serum ferritin levels were significantly decreased among infected gerbils at both the 11-week and 16-week time points ($1,004.0 \pm 379.4$ ng/ml and 516.5 ± 123.8 ng/ml, respectively) compared to those among uninfected gerbils ($2,036.0 \pm 341.4$ ng/ml) ($P < 0.05$) (Fig. 2A). The serum ferritin levels among infected gerbils diagnosed with microcytic anemia were significantly decreased compared to the serum ferritin levels among infected gerbils determined to be nonanemic, infected gerbils with normocytic anemia, and uninfected gerbils ($P < 0.05$) (Fig. 2B). Infected gerbils maintained on a low-iron diet or a combination of

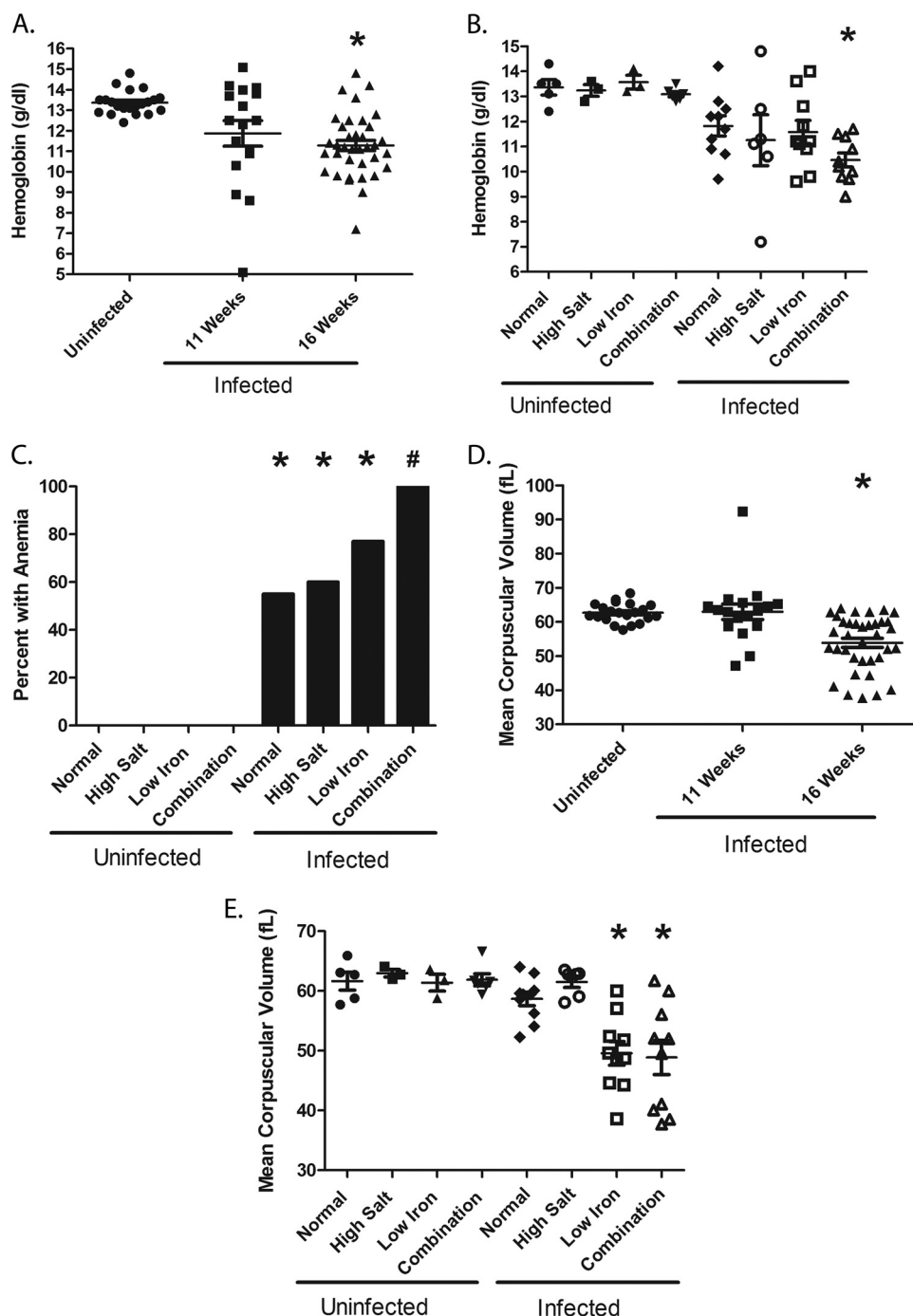


FIG 1 Reduced hemoglobin levels in *H. pylori*-infected gerbils. Horizontal bars represent means \pm SEMs, and asterisks represent significant differences ($P < 0.05$). (A) Hemoglobin levels are shown for uninfected gerbils (the results for the 11- and 16-week time points combined), infected gerbils at 11 weeks postinfection, and infected gerbils at 16 weeks postinfection. Hemoglobin levels in infected gerbils (at 16 weeks postinfection) were significantly reduced compared to those in the uninfected group ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison). (B) Hemoglobin levels at the 16-week time point, by diet. Hemoglobin levels in infected gerbils on a combination of a high salt and low-iron diet differed significantly from the levels in the uninfected cohort on a regular diet (*, $P < 0.05$, Kruskal-Wallis test with Dunn's multiple-comparison test) or the combination high-salt and low-iron diet ($P < 0.05$). (C) Percentage of gerbils considered anemic at the 16-week time point, by diet. The incidence of anemia was higher in each of the infected cohorts than the entire group of uninfected gerbils (* and #, $P < 0.005$, Fisher's exact test). When various infected cohorts were compared to the infected cohort on a normal diet, only the infected gerbils on a high-salt/low-iron diet had a significantly higher incidence of anemia (#, $P = 0.02$). (D) The MCV for uninfected gerbils (the results for the two time points combined), infected gerbils at 11 weeks postinfection, and infected gerbils at 16 weeks postinfection is shown. The MCVs of infected gerbils at 16 weeks postinfection were significantly lower than the MCVs of uninfected gerbils or infected gerbils at 11 weeks postinfection ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple-comparison test). (E) MCVs at the 16-week time point, by diet. When each cohort is compared to the reference group of uninfected gerbils on a normal diet, the MCVs of infected gerbils on a low-iron diet and the MCVs of infected gerbils on a high-salt/low-iron diet were significantly lower ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple-comparison test). These two groups also had significantly reduced MCVs compared to uninfected groups on the same diets ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple-comparison test).

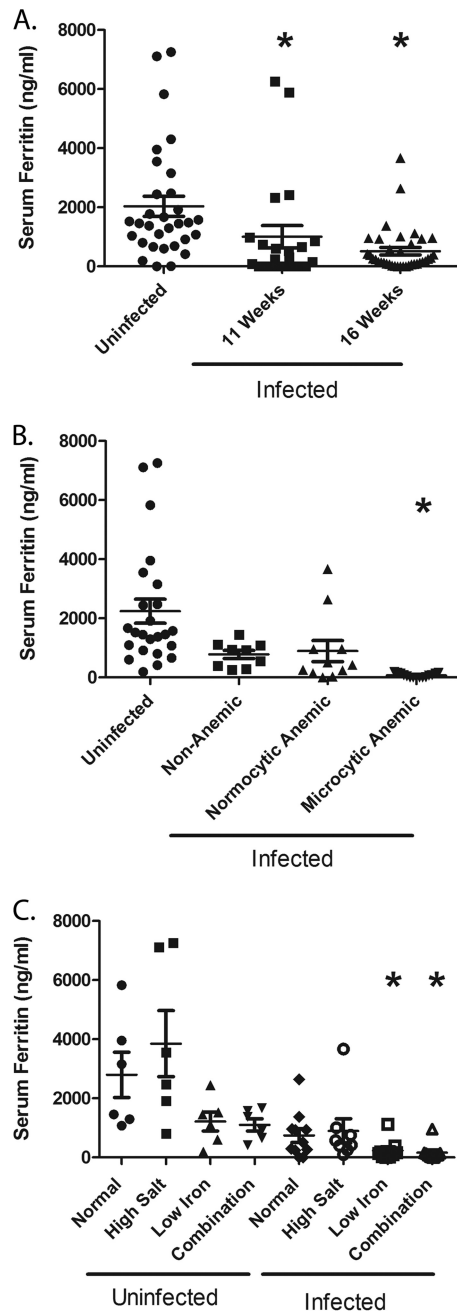


FIG 2 Reduced serum ferritin levels in *H. pylori*-infected gerbils. Horizontal bars represent means \pm SEMs, and asterisks represent significant differences ($P < 0.05$). (A) Serum ferritin levels are shown for uninfected gerbils (the results for the two time points combined), infected gerbils at 11 weeks postinfection, and infected gerbils at 16 weeks postinfection. Infected gerbils (both time points) had significantly reduced serum ferritin levels compared to the uninfected gerbils ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple-comparison test). (B) Serum ferritin levels at the 16-week time point, by anemia status. The serum ferritin levels in infected gerbils diagnosed with microcytic anemia (69.66 ± 17.5 ng/ml) were significantly decreased compared to the serum ferritin levels in infected gerbils determined to be nonanemic (780.3 ± 139.5 ng/ml), infected gerbils with normocytic (normal MCV) anemia (894 ± 357.7 ng/ml), and uninfected gerbils ($2,243 \pm 405.5$ ng/ml) ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple-comparison test). (C) Serum ferritin levels of gerbils, by diet at the 16-week time point. Infected gerbils on a low-iron diet and infected gerbils on a combination of a high-salt and low-iron diet had significantly lower serum ferritin values than the uninfected gerbils on a normal diet (236.1 ± 117.3 ng/ml, 165.2 ± 89.6 ng/ml, and $2,796 \pm 767.5$

a high-salt and low-iron diet exhibited the greatest reductions in ferritin compared to the uninfected gerbils on a normal diet ($P < 0.05$) (Fig. 2C; see also Fig. S1F in the supplemental material) and also had significantly reduced serum ferritin values compared to the uninfected gerbils on the same diets (Fig. 2C).

To determine if IDA was present in individual gerbils, we developed criteria for iron deficiency based on serum ferritin values, as described in Materials and Methods. If a gerbil was deemed to be both anemic and iron deficient, the gerbil was classified as having IDA. By these criteria, IDA occurred in 57% of all infected gerbils and none of the uninfected animals ($P < 0.001$, Fisher's exact test). *H. pylori*-infected gerbils on a combination high-salt and low-iron diet had a significantly higher rate of IDA (90%) than infected gerbils receiving the normal diet (30%) ($P = 0.0163$, Fisher's exact test). Collectively, these data indicate that iron deficiency anemia occurs more commonly in *H. pylori*-infected gerbils than in uninfected gerbils. Moreover, a diet containing an increased salt content and a decreased iron content promoted the development of iron deficiency anemia in *H. pylori*-infected gerbils but not in uninfected gerbils during the time course of these experiments.

Gastric ulceration, gastric cancer, and gastric inflammation in *H. pylori*-infected gerbils. One possible mechanism by which iron deficiency can arise is through chronic blood loss. To detect gastrointestinal blood loss, we tested stool samples collected at the 16-week time point for the presence of fecal occult blood. Fecal occult blood was detected at a significantly higher frequency in the infected gerbils than in the uninfected gerbils (68.57% and 4.76% of gerbils were positive for occult blood, respectively; $P < 0.0001$, Fisher's exact test). *H. pylori*-induced gastric ulceration represents a potential source of the blood found in the fecal contents. To detect gastric ulceration, gastric histologic sections were examined as described in Materials and Methods. No gastric ulcers were observed in uninfected gerbils (Fig. 3A), whereas gastric ulcers were detected in 46% of infected gerbils at the 16-week time point. Ulcers were predominantly located either in the transitional mucosa between the corpus and antrum or in the proximal two-thirds of the antrum (Fig. 3B). Among the infected gerbils, there was no correlation between the presence of gastric ulcers and the detection of fecal occult blood. The detection of fecal occult blood in a high proportion of infected gerbils without gastric ulcers is consistent with a limited sensitivity of the histologic methodology used for detection of gastric ulcers. The methodology used to assess fecal occult blood also has limitations. For example, detection of fecal occult blood may be insensitive if blood loss from gastric ulcers is intermittent.

As expected, infected, ulcerated gerbils had significantly lower hemoglobin levels than infected, nonulcerated gerbils (10.3 ± 0.3 g/dl and 12.0 ± 0.3 g/dl, respectively; $P = 0.0002$) (Fig. 4A). Concordantly, gastric ulceration was detected in 62% (13 out of 21) of gerbils diagnosed as having IDA, whereas it was detected in only 14% (2 out of 14) of infected gerbils that did not have IDA ($P = 0.0062$) (Fig. 4B). Among infected gerbils, those on a diet containing increased salt (either the high-salt diet or the combination of a

ng/ml, respectively; $P < 0.05$ Kruskal-Wallis test with Dunn's multiple-comparison test). The ferritin levels in the first two groups also differed significantly from the ferritin levels in the uninfected cohorts on the same diets ($P < 0.05$).

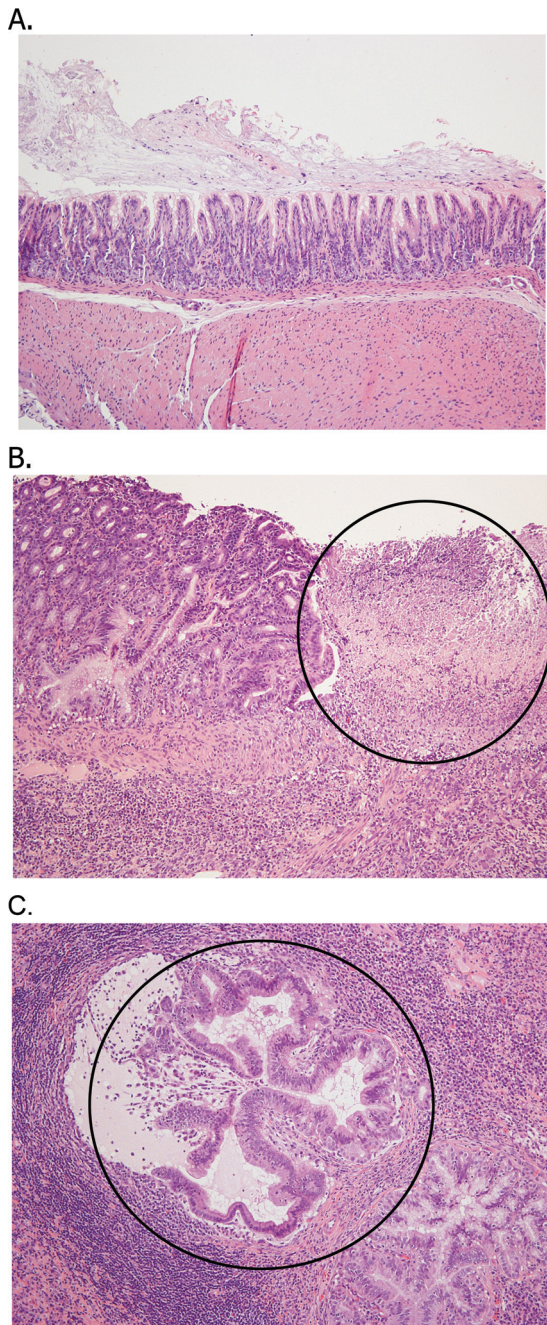


FIG 3 Gastric ulceration and gastric cancer in infected gerbils. (A) Gastric mucosa of an uninfected gerbil on a normal diet. (B) Gastric mucosa of an infected gerbil on a high-salt diet. A gastric ulcer in the proximal third of the antrum is visible in the upper right portion of the image and is circled. (C) Invasive gastric adenocarcinoma (circled) in an infected gerbil on a high-salt diet, characterized by irregular glands infiltrating the submucosa. Magnifications, $\times 100$.

high-salt and low-iron diet) had a significantly higher incidence of gastric ulceration (ulceration in 12 of 19 animals [63%]) than gerbils not on a high-salt diet (ulceration in 6 of 20 animals on a normal or a low-iron diet [30%]) ($P = 0.039$) (Fig. 4C).

Another possible cause of blood loss and anemia is the development of gastric adenocarcinoma (Fig. 3C). At the 16-week time

point, gastric adenocarcinoma was detected in 72% (28 out of 39) of *H. pylori*-infected gerbils and none of the uninfected gerbils. Gastric ulcers were detected in 64% of the 28 gerbils with adenocarcinoma and in none of the 11 *H. pylori*-infected gerbils without adenocarcinoma. Thus, gerbils exhibiting a gastric ulcer were significantly more likely than the infected, nonulcerated cohort of gerbils to be diagnosed as having gastric adenocarcinoma ($P = 0.0002$) (Fig. 4B). To determine if gastric cancer was a cause of blood loss and anemia independently of gastric ulceration, we classified the infected nonulcerated gerbils into two subgroups on the basis of the presence or absence of gastric cancer (Fig. 4A). No significant difference in hemoglobin levels was detected between these two nonulcerated subgroups, suggesting that the anemia was mainly attributable to gastric ulceration instead of gastric cancer. Most of the gastric adenocarcinomas detected in this study were confined to the mucosa and did not penetrate through the gastric epithelium, which probably explains why the presence of gastric cancer was not an independent cause of anemia.

As expected, gastric inflammation was present in *H. pylori*-infected gerbils but not uninfected gerbils ($P < 0.0001$), as determined by gastric histology scores (see Fig. S2A in the supplemental material). The severity of gastric inflammation was associated with markers of IDA (decreased serum ferritin and hemoglobin levels) at the 16-week time point ($P < 0.0001$) (see Fig. S2B and C), but this relationship was not statistically significant if uninfected animals were excluded from the analysis (i.e., if only infected animals were analyzed).

Anemia and gastric pH. Increased gastric pH (hypochlorhydria) can lead to decreased iron absorption, iron deficiency, and anemia (56). Therefore, we examined the relationship between gastric pH and hematological alterations. In animals harvested at both 16 weeks and 11 weeks postinfection, gastric pH values were significantly higher in *H. pylori*-infected gerbils (4.4 ± 0.2 and 3.7 ± 0.1 , respectively) than in uninfected gerbils (3.0 ± 0.0) ($P < 0.05$) (Fig. 4D). There was an inverse association between gastric pH and hemoglobin values ($P < 0.0001$) (see Fig. S3A in the supplemental material). Similarly, there was an inverse association between gastric pH and serum ferritin values ($P < 0.0001$) (see Fig. S3B). These relationships were not statistically significant if uninfected animals were excluded from the analyses (i.e., if the analyses were limited to only infected gerbils).

Consistent with previous reports (33), increased gastric pH was associated with increased gastric inflammation ($P < 0.0001$) (see Fig. S2D in the supplemental material). This relationship was statistically significant even when uninfected animals were excluded from the analysis. Previous studies have shown that *H. pylori* infection can lead to the loss of parietal cells (acid-secreting cells found in the stomach) in Mongolian gerbils as well as atrophic gastritis and parietal cell loss in humans (33, 57). Similarly, infected gerbils in the current study exhibited parietal cell loss, whereas uninfected gerbils did not (Fig. 5; see also Fig. S3E). A reduction in chief cells was also detected in infected gerbils compared to uninfected gerbils ($P < 0.0001$) (Fig. 5; see also Fig. S3F). As expected, both parietal cell loss and chief cell loss were positively correlated with gastric pH, suggesting that parietal cell loss was the cause of hypochlorhydria ($P < 0.0001$) (see Fig. S3C). In addition, parietal cell loss was inversely correlated with hemoglobin levels ($P < 0.0001$) (see Fig. S3D). In summary, gastric inflammation and ulceration, parietal cell loss, and increased gastric pH

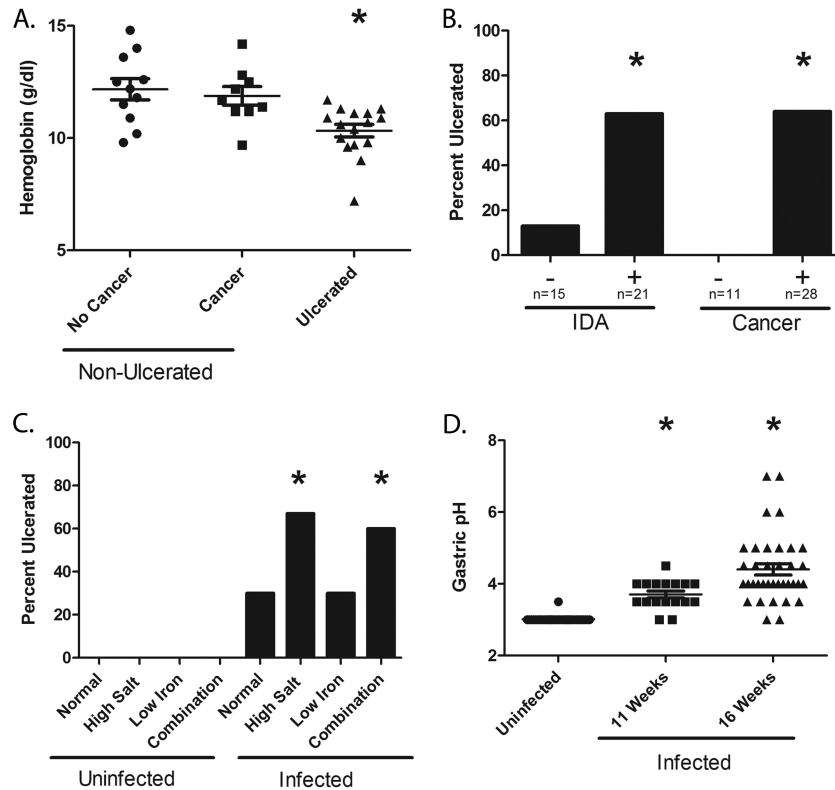


FIG 4 Relationship between anemia and gastric ulceration. Horizontal bars represent means \pm SEMs, and asterisks represent significant differences ($P < 0.05$). (A) Hemoglobin levels of infected gerbils harvested at the 16-week time point were stratified by cancer and ulceration status. All ulcerated gerbils had gastric cancer. Hemoglobin levels were significantly reduced in infected gerbils with gastric ulcers compared to infected gerbils without gastric ulceration (either with concomitant gastric cancer or without evidence of gastric cancer) ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple-comparison test). (B) Incidence of gastric ulceration at the 16-week time point in infected gerbils with or without IDA, and incidence of gastric ulceration in infected gerbils with or without gastric cancer. Gerbils with IDA or gastric cancer had a significantly higher incidence of gastric ulceration than gerbils without IDA or gerbils without cancer, respectively ($P = 0.0062$ and $P = 0.0002$, respectively, Fisher's exact test). (C) Percentage of gerbils with a gastric ulcer at the 16-week time point, by diet. Asterisks represent a significant difference ($P < 0.05$) when combined data from cohorts consuming diets high in salt (high salt and combination) are compared to combined data from uninfected animals. (D) The gastric pH of uninfected gerbils (at both time points), infected gerbils at the 11-week time point, and infected gerbils at the 16-week time point is presented. The gastric pH was significantly higher in both infected cohorts than in the uninfected cohort ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple-comparison test).

were each correlated with the development of iron deficiency anemia and likely contributed to the development of this disorder.

Analysis of leukocytes and platelets. In addition to analyzing the effects of *H. pylori* infection on the development of anemia, we investigated the effects of *H. pylori* and diet on other hematologic parameters. At the 16-week time point, infected gerbils had significantly elevated white blood cell (WBC) counts compared to uninfected gerbils ($14.0 \times 10^9 \pm 1.1 \times 10^9/\text{liter}$ and $9.8 \times 10^9 \pm 0.7 \times 10^9/\text{liter}$, respectively; $P = 0.009$) (Fig. 6A). Consistent with the elevated total WBC counts, neutrophil counts and monocyte counts were significantly elevated in infected cohorts compared to uninfected gerbils ($P = 0.001$) (Fig. 6B and C). When infected anemic gerbils were compared with infected nonanemic gerbils, there was no difference in total WBC counts or neutrophil counts (Fig. 6A and B), but infected anemic gerbils had significantly elevated monocyte counts ($1.3 \times 10^9 \pm 0.3 \times 10^9/\text{liter}$) compared to the infected nonanemic cohort ($0.3 \times 10^9 \pm 0.04 \times 10^9/\text{liter}$, $P < 0.05$) (Fig. 6C).

Infected gerbils had significantly higher platelet counts than uninfected gerbils ($1,082.0 \times 10^9 \pm 49.2 \times 10^9/\text{liter}$ and $732.5 \times 10^9 \pm 18.9 \times 10^9/\text{liter}$, respectively; $P < 0.0001$) (Fig. 6D). A

positive correlation between platelet count and gastric pH ($P < 0.0001$) (see Fig. S4A in the supplemental material) and between platelet count and gastric ulcers ($P = 0.013$) (data not shown) was observed. Increased platelet counts were associated with several markers of iron deficiency anemia. Specifically, platelet counts were inversely correlated with hemoglobin levels ($P < 0.0001$), mean corpuscular volume ($P = 0.001$), and serum ferritin levels ($P = 0.013$) (see Fig. S4B to D). Taken together, these results indicate that leukocytosis and thrombocytosis occur commonly in *H. pylori*-infected gerbils and that monocytosis occurs more commonly in *H. pylori*-infected gerbils with IDA than in infected non-anemic gerbils.

DISCUSSION

In this study, we demonstrate that *H. pylori* infection causes IDA in the Mongolian gerbil model. Previous studies in rodent models have reached inconsistent conclusions about the capacity of *H. pylori* to cause anemia (19–22). The inconsistent conclusions might reflect the use of different *H. pylori* strains, differences in animal models, differences in diets, or differences in the time points selected for analysis. In the current study, we used *H. pylori*

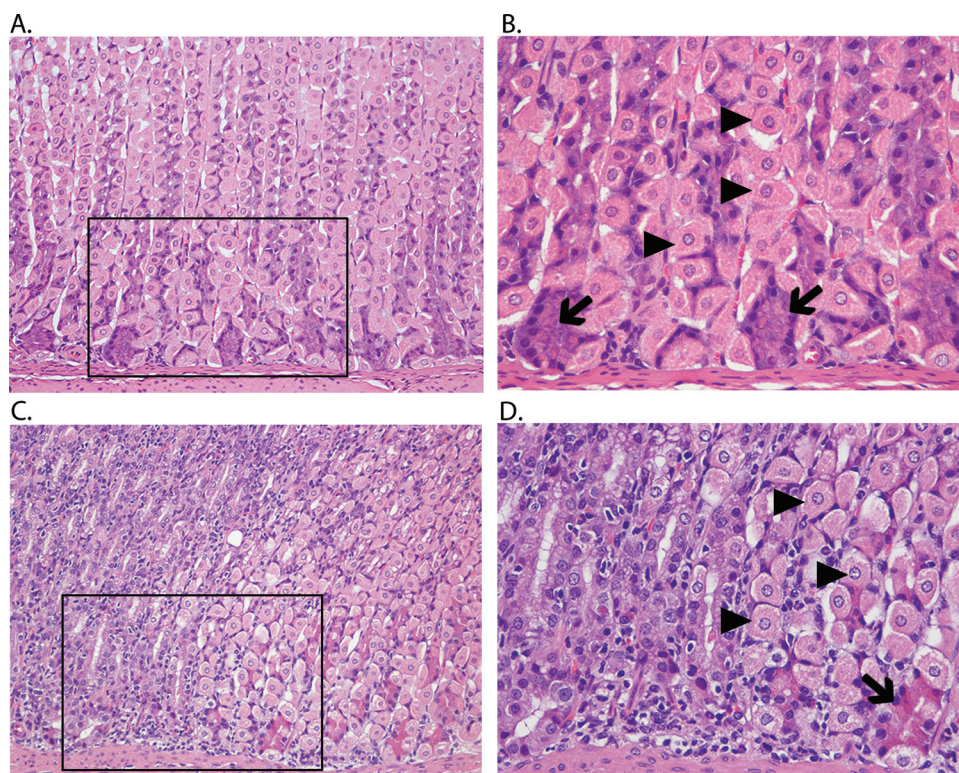


FIG 5 Hematoxylin- and eosin-stained sections of gerbil corpus mucosa. Chief cells (arrows) are basophilic and mainly located at the base of the glands. Parietal cells (arrowheads) are large and eosinophilic and display a triangular or round shape. (A and B) Normal mucosa. (C and D) An *H. pylori*-induced loss of parietal and chief cells is observed on the left side of the images. These cells have been replaced by antral-type epithelial cells. The boxed areas in panels A and C are magnified in panels B and D, respectively. Magnifications $\times 200$ (A and C) and $\times 400$ (B and D).

strain 7.13, which produces CagA and has a functional *cag* type IV secretion system. A previous study infected Mongolian gerbils with a CagA-positive strain and did not detect anemia in the infected gerbils (22). The reason for the difference in results is not known, but we speculate that the animals in the current study may have had more severe gastric pathology than the animals in the previous study. A direct comparison of gastric histologic features in these studies is not possible because the previous study did not examine gastric tissue for the presence of ulceration, cancer, or inflammation (22).

There are multiple potential mechanisms by which *H. pylori* infection could lead to anemia. Chronic gastrointestinal blood loss is a well-known cause of iron deficiency anemia, and in the current study, we detected gastric ulceration and gastrointestinal blood loss in a high proportion of the *H. pylori*-infected animals. In addition, we detected increased gastric pH and parietal cell loss in *H. pylori*-infected animals. Therefore, both blood loss from gastric ulceration and *H. pylori*-induced hypochlorhydria (with the resulting impaired iron absorption) probably contributed to the development of anemia (56).

We also examined the effects of diet on *H. pylori*-induced IDA and found that *H. pylori*-infected animals fed a high-salt/low-iron diet had a higher incidence and an increased severity of IDA compared to *H. pylori*-infected animals receiving a regular diet. Importantly, neither the low-iron diet nor the high-salt diet resulted in anemia in uninfected animals. Over a long period of time, a low-iron diet would eventually be expected to result in IDA in uninfected animals (1). Presumably, the time course of the exper-

iments in the current study was not long enough to permit the development of IDA in uninfected animals on a low-iron diet. One interpretation is that *H. pylori* infection accelerates or exacerbates the development of IDA in response to a low-iron diet (and, possibly, a high-salt diet). In addition, a low-iron diet or a high-salt diet might augment the capacity of *H. pylori* infection to cause IDA.

In support of the latter model, previous studies have shown that administration of diets high in salt or low in iron to *H. pylori*-infected gerbils results in an increased severity of gastric inflammation and an increased incidence of gastric cancer (33, 35, 37). It has been proposed that these effects are attributable to enhanced *H. pylori* virulence under high-salt or low-iron conditions (33, 35, 44, 46, 47, 58). For example, a high-salt environment stimulates increased production of the *H. pylori* effector protein CagA, and a low-iron environment stimulates enhanced activity of the *H. pylori* *cag* T4SS (which is required for the entry of CagA into gastric epithelial cells) (35, 44, 45, 58). Infection of animals with CagA-positive strains that have a functional *cag* type IV secretion system results in increased gastric inflammation and damage compared to infection of animals with *cagA* mutant strains (33, 35) or mutant strains that have defects in *cag* T4SS activity (59, 60). Therefore, increased production of CagA in response to high-salt conditions and increased delivery of CagA into host cells in response to low-iron conditions are likely mechanisms by which these dietary alterations influence the development of a gastric pathology.

In contrast to previous studies that reported elevated rates of gastric cancer in *H. pylori*-infected gerbils receiving a high-salt or

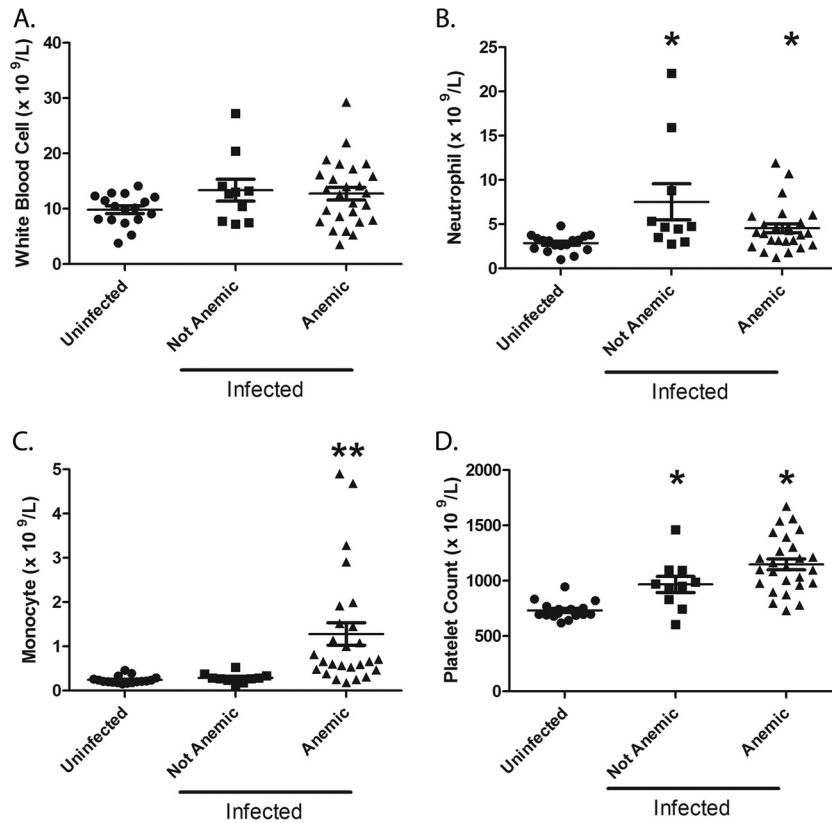


FIG 6 Leukocytosis and thrombocytosis in *H. pylori*-infected gerbils. Horizontal bars represent means \pm SEMs, and asterisks represent significant differences ($P < 0.05$). Total white blood cell counts, neutrophil counts, monocyte counts, and platelet counts in uninfected gerbils, infected gerbils without anemia, and infected anemic gerbils were determined at the 16-week time point. (A) Total WBC counts did not differ among the groups ($P = 0.21$, Kruskal-Wallis test with Dunn's multiple-comparison test). (B) Neutrophil counts were significantly elevated in the infected cohorts compared to the uninfected gerbils ($P \leq 0.001$, Kruskal-Wallis test with Dunn's multiple-comparison test). (C) The monocyte counts in the infected anemic (based on hemoglobin values) cohort were significantly higher than the monocyte counts in the uninfected cohort or the infected nonanemic cohort ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple-comparison test). (D) Platelet counts of gerbils in both infected cohorts (anemic or not anemic) were significantly higher than those in the uninfected cohort ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple-comparison test).

a low-iron diet (33, 35), the composition of the diet did not influence gastric cancer rates in the current study. Notably, the gastric cancer rates in infected animals on a regular diet were considerably higher in this study than in previous studies (33, 35). When the methods used in the various studies are compared to those used in the present study, one difference pertains to the type of diet fed to the control animals. In the current study, the control group received AIN-93M chow (which contains 0.26% sodium chloride and 39 ppm iron) and the other groups received modified forms of this diet. In contrast, the control groups in previous studies received either a modified form of AIN-93M chow (containing 250 ppm iron) or a Purina 5001 diet (which contains 0.75% sodium chloride and 240 ppm iron) (33, 35).

We propose that there may be a positive-feedback loop, whereby low-iron or high-salt conditions lead to the increased delivery of secreted *H. pylori* virulence factors, such as CagA (33, 35, 44), resulting in gastrointestinal blood loss and impaired iron absorption, and that the consequent iron deficiency drives the continued enhanced production and delivery of *H. pylori* virulence factors (Fig. 7). In addition, we speculate that the inflammatory environment generated by a high-salt or a low-iron diet drives the selection of *H. pylori* strains that produce multiple vir-

ulence factors and are thereby most fit for growth in an inflammatory environment (61).

In addition to detecting anemia in *H. pylori*-infected gerbils, we detected several other hematologic abnormalities, including leukocytosis and thrombocytosis. These findings indicate that *H. pylori* colonization of the gerbil stomach leads to not only a gastric mucosal inflammatory response but also a systemic response. Previous studies reported that humans infected with *H. pylori* have significantly higher leukocyte counts than their uninfected counterparts (62, 63). The leukocytosis observed in *H. pylori*-infected gerbils in the current study was substantially greater than the leukocytosis previously observed in *H. pylori*-infected humans. We speculate that the prolonged coevolution of *H. pylori* and humans has favored the selection of *H. pylori*-host interactions characterized by a minimal systemic host response (64, 68). As gerbils are not a natural host for *H. pylori*, this could explain the exaggerated systemic inflammatory response observed in the current study.

The thrombocytosis detected in the current study occurred in a large proportion of animals, presumably as part of a systemic inflammatory response to *H. pylori* infection. This is markedly different from ITP, which occurs in only a very small proportion of *H. pylori*-infected humans through a different immunologic

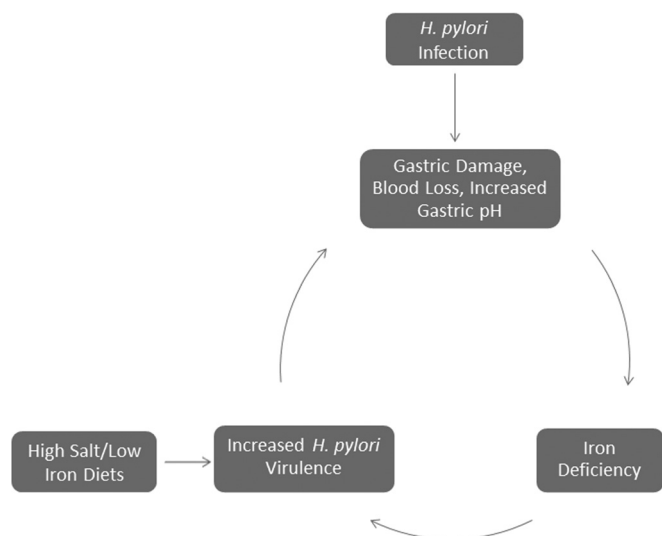


FIG 7 Positive-feedback loop for *H. pylori*-induced gastric damage and iron deficiency anemia. *H. pylori* infection causes gastric damage, gastrointestinal blood loss, and increased gastric pH (resulting in impaired iron absorption), all of which can result in iron deficiency. Consumption of a high-salt or a low-iron diet enhances *H. pylori* virulence through multiple mechanisms, including increased production of CagA and increased activity of the *cag* T4SS. Therefore, high-salt or low-iron diets accelerate or exacerbate the *H. pylori*-induced gastric alterations that lead to iron deficiency. The worsening iron deficiency provides an ongoing stimulus for enhanced *H. pylori* virulence, leading to continued gastric damage and blood loss.

mechanism. The increased platelet counts occurring in association with IDA have been reported in other rodent models of IDA, as well as in humans with IDA (65, 66).

Unexpectedly, we detected markedly elevated monocyte counts in *H. pylori*-infected gerbils with anemia compared to infected gerbils without anemia. It has previously been suggested that under conditions of iron deficiency, apoptotic signaling pathways in some hematopoietic lineages are rendered nonfunctional, resulting in a longer life span for white blood cells, such as monocytes and neutrophils (67). Delayed or absent apoptotic processes could potentially account for the increased circulating monocytes observed in the anemic animals in this study, as this would extend the lifetime of these cells (67).

In summary, these data provide strong evidence indicating that *H. pylori* can cause iron deficiency anemia and a systemic inflammatory response in an animal model. Gastrointestinal blood loss from *H. pylori*-induced gastric ulceration is probably the main cause of the anemia, but reduced iron absorption as a result of increased gastric pH may also be contributory. High-salt and low-iron diets enhanced the capacity of *H. pylori* to cause gastric ulceration and/or iron deficiency anemia but had no detectable effect on uninfected animals. These findings highlight the important role of diet as a factor influencing the outcome of *H. pylori* infection.

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