Quantitative evaluation of inflammatory and immune responses in the early stages of chronic Helicobacter pylori infection

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Quantitative Evaluation of Inflammatory and Immune Responses in the Early Stages of Chronic Helicobacter pylori Infection

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The early consequences of Helicobacter pylori infection and the role of bacterial virulence determinants in disease outcome remain to be established. The present study sought to measure the development of host inflammatory and immune responses and their relationship to the putative bacterial virulence factors cag pathogenicity island (cagPAI), vacA allele, and oipA in combination with bacterial colonization density in a feline model of the early stages of H. pylori infection. Gastric tissues obtained from infected and uninfected cats were evaluated for H. pylori ureB, cagPAI, vacA allele, and oipA and colonization density (urease, histology, and real-time PCR). Inflammation was assessed by measuring mRNA upregulation of interferon (IFN-γ), interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, and IL-12 p40 and histopathology. The mucosal immune response was characterized by morphometric analysis of lymphoid follicles and by differentiating lymphocyte populations with antibodies against surface markers. Infecting H. pylori strains were positive for vacA S1 but lacked cagPAI and an active oipA gene. Colonization density was uniform throughout the stomach. Upregulation of IFN-γ, IL-1α, IL-1β, and IL-8 and increased severity of inflammatory infiltrates and fibrosis were observed in infected cats. The median number and total area of lymphoid aggregates were 5 and 10 times greater, respectively, in the stomachs of infected than uninfected cats. Secondary lymphoid follicles in uninfected cats were rare and positive for BLA36 and B220 but negative for CD3 and CD79α, whereas in infected cats they were frequent and positive for BLA36, CD79α, and CD3 but negative for B220. Upregulation of IFN-γ, IL-1α, IL-1β, and IL-8 and marked hyperplasia of secondary lymphoid follicles are early consequences of H. pylori infection in cats. The response appears to be similar to that of infected people, particularly children, can develop independently of the pathogenicity factors cagPAI and oipA, and is not correlated with the degree of colonization density or urease activity.

Helicobacter pylori is a gram-negative bacterium that chronically infects more than half of all people worldwide (6, 66, 69, 75). Infection tends to begin in infancy (new infections are uncommon in adults) and is likely to last for decades (16).

Over a span of 20 to 30 years, about one in six H. pylori-infected adults in the West develops duodenal ulcers that are associated with antral predominant gastritis and increased acid secretion (18). Another smaller subset of people develop atrophic and intestinal metaplasia of the body of the stomach and go on to develop gastric carcinoma over a period of 30 to 40 years (55). While the factors determining this variable outcome are not well understood, the development of a sustained gastric atrophy and intestinal metaplasia of the body of the stomach and the inflammation of polymorphonuclear and mononuclear cells and the upregulation of proinflammatory cytokines and the chemokine interleukin-8 (IL-8) (44, 57). Mucosal T cells in infected individuals are polarized toward the production of gamma interferon (IFN-γ) rather than IL-4 or IL-5, indicating a strong bias toward a T₄₁ type response (5, 44).

Bacterial determinants of virulence are considered critical for initiating close interactions with host epithelial cells and inducing mucosal inflammation. H. pylori strains containing the cytotoxicity-associated gene pathogenicity island (cagPAI), are associated with more severe antral inflammation, higher mucosal levels of IL-1α, IL-1β, and IL-8 (57), peptic ulceration (72), and increased acid secretion (18). A number of cagPAI-encoded proteins such as cagE but not cagA help to induce tyrosine kinase phosphorylation of host cell signaling factors and activation of NF-κB and IL-8 secretion in vitro (43, 62). Thus, it has become widely accepted that IL-8 induction in response to colonization with cagPAI+ H. pylori is a key step in the pathogenesis of neutrophilic gastritis observed in adults with H. pylori infection. However, cagPAI− strains with similar abilities to induce IL-8 in vitro are associated with markedly different outcomes in vivo (79), and the presence or absence of the cagPAI or its marker cagA do not reliably predict the outcome of H. pylori infection in people (7, 28, 36). The recent identification of a non-cagPAI-encoded outer inflammatory protein (OipA) involved in the upregulation of IL-8 in vitro and disease outcome in people (80) and the great genetic diversity of H. pylori as a species (12) may help to clarify part of this discord.

Other virulence-independent factors such as the duration...
and density of infection may also influence the outcome but have been less well studied. Surveys of the early phases of infection, i.e., in children, are few in general but indicate that a proinflammatory response and TNF-α-type cytokines were detected in response to infection (30, 45). In contrast to adults, in whom neutrophilic infiltrates predominate, children develop a predominantly mononuclear infiltrate and have a higher degree of lymphoid follicular hyperplasia (15, 33, 38, 51, 52, 76). Infection in children is also rarely associated with severe disease (17, 51, 52), and colonization with strains considered virulent in adults does not correlate with the severity of endoscopic and histologic findings (17, 27). The mechanisms underlying the differences in histopathology and disease pattern between children and adults and the early and late stages of infection are still not well understood (15, 27, 33, 38, 51, 52).

A variety of model systems have been developed to overcome some of the difficulties, e.g., genetic heterogeneity of *H. pylori* and the host organisms, inherent in studying patients presenting with spontaneous disease (40). We have been drawn to the domestic cat model of *H. pylori* infection because cats are susceptible to natural or experimental infection (22) and their gastric anatomy, physiology, immunology, and overall genetic makeup are more closely aligned with humans than are those of rodents (46, 68). Unlike mice, cats are able to produce disease-relevant factors, especially IL-8 (64), and they are relatively nonresponsive to vaccination strategies with orally administered *H. pylori* surface antigens that are effective in mice (40). Due to their size, cats can also be studied repeatedly by endoscopy. However, a lack of species-specific reagents and quantitative techniques to study immune and inflammatory responses and limited knowledge of the genotypes of *H. pylori* strains infecting cats have restricted investigations to date.

Here we report the quantitative evaluation of the gastric mucosal inflammatory and immune responses during the early stages of chronic *H. pylori* infection and their relationship to *cag*PAI, *vacA* allele, *oipA*, and bacterial colonization density. We also further define the lymphoid hyperplasia that develops in response to infection by characterization with surface markers.

**Materials and Methods**

**Animals.** Five clinically healthy cats (one female, four males; all domestic shorthairs) that were born into our *H. pylori*-infected colony were studied. This colony is chronically infected with *H. pylori* and is free from the large gastric spiral helicobacters *H. felis* and *H. heilmannii*, as determined by species-specific PCR and histology (20, 64). The five cats were known to have acquired *H. pylori* infection by 8 to 14 weeks of age (15, 33, 51, 52), and colonization with strains considered virulent in adults does not correlate with the severity of endoscopic and histologic findings (17, 27). The mechanisms underlying the differences in histopathology and disease pattern between children and adults and the early and late stages of infection are still not well understood (15, 27, 33, 38, 51, 52, 76).

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**Endoscopic biopsy.** Endoscopic biopsy samples of the stomach were obtained from each cat with a video gastroscopy and biopsy forceps (Fujinon EG7FP2, Tech-Star Medical Inc., Spring Hill, Fla.) to confirm their infection status prior to inclusion in the study. Endoscopy was performed in kittens born into our *H. pylori*-infected colony between 8 and 14 weeks of age. The endoscope was thoroughly cleaned and then sterilized with a solution consisting of hydrogen peroxide and phosphoric acid (Sporox, Mohawk Hospital Equipment, Utica, N.Y.). The biopsy cups were finally immersed in Chlorox (1:10 in water) for 10 min to destroy residual DNA.

**Gastric biopsy.** For histopathology studies, samples were obtained from all cats at necropsy with sterile 6-mm skin biopsy punches. Samples from the cardia, mid-fundus, and mid-pylorus were evaluated for the presence of *Helicobacter* DNA by PCR. A further two samples were obtained for cytology analysis, snap frozen in liquid nitrogen, and stored at −80°C pending analysis. A full-thickness strip of stomach along the greater curvature, cut from the cardia to pylorus, was bathed in the pylorus innermost and secured with a needle (20 gauge), fixed in 10% buffered formalin for 24 h and used for the evaluation of histopathology, histomorphometry, and immunohistochemistry.

**Urease activity.** Urease activity of gastric tissue was evaluated as previously described (58). In short, gastric mucosal urea biopsy samples were placed in sterile tubes containing 200 μl of a solution composed of urea, sodium azide, phenol red, and phosphate-buffered saline (pH 6.5). The biopsy specimens were incubated for 24 h, and any change in color of the indicator medium was scored at 2, 4, 8, 12, and 24 h. A change from orange to pink was considered a positive result, and the time of the color change was recorded. Urease results were scored as follows: positive at 2 h = 5, positive at 4 h = 4, positive at 8 h = 3, positive at 12 h = 2, positive at 24 h = 1, and negative at 24 h = 0.

**Histology.** Formalin-fixed gastric mucosal strips were embedded in paraffin and sectioned at 4 to 6 μm. Serial sections of each block were stained with modified Steiner’s stain (25). Samples were evaluated according to site (cardia, fundus/body, and pyloric antrum) in a blinded fashion by one pathologist (E.S.) for the presence of *Helicobacter*-like organisms (HLO) and degree of colonization. The degree of colonization by HLO was graded as follows: 0 = no HLO seen; +1 = presence of HLO in <5% of gastric glands; +2 = presence of HLO in 5 to 50% of gastric glands; and +3 = presence of HLO in >50% of gastric glands.

**PCR.** In order to identify *H. pylori* in gastric biopsy samples, tissue samples were frozen at −80°C. DNA was extracted with a Qiamp tissue kit (Qiagen, Santa Clarita, Calif.). PCR was performed with primers that amplify the urease B gene of *H. pylori* (Table 1) (54) and with *Helicobacter* genus-specific primers directed against 16S rRNA, as described previously (23, 67). The relatedness of *H. pylori* strains in kittens and their mothers was evaluated by restriction fragment length polymorphism analysis of *H. pylori* ureB amplicons (2). The PCR products were precipitated with 100% ethanol and sodium acetate, the pellets were washed with 70% ethanol, dried in a desicator, and resuspended with sterile Tris-EDTA buffer. The concentrated PCR product (10 μl) was digested with 10 U of the restriction enzyme HaellIII in a buffer for 2 h at 37°C according to the manufacturer’s protocol (Roche Diagnostics Corp., Indianapolis, Ind.). The digested samples were analyzed by electrophoresis in 1.5% agarose gels. *Helicobacter* reference strains from humans (ATCC 49504, 43504, and 8826) were used for comparison.

**DNA samples from *H. pylori*-infected cats were negative for *H. felis* and *H. heilmannii* DNA by conventional PCR (54).**

**Gene-specific PCR to test for the presence or absence of the virulence-associated *cag*PAI, *cag*PAI (with primers directed against the cag empty site [I]), *oipA*, and *vacA* allele (Table 1) was carried out with 1 μl of extracted DNA in 50-μl volumes of Qiagen mastermix (Qiagen) and 500 nM primer concentration (see Table 1 for primer sequences) for 30 (cagPAI and vacA) or 40 (oipA) cycles under the following conditions: 2 min at 94°C, 2 min at 55°C for *oipA*, and 72°C for a time chosen based on the size of the expected fragment (1 min/kbp).**

**PCR amplicons were visualized with ethidium bromide in agarose gels. Sequencing of *oipA* PCR products was performed to determine if the gene was in the on or off position (80).** PCR products (without cloning) were isolated from agarose gels with the QIAquick PCR purification kit (Qiagen Inc.) and sequenced with an ABI 377 automated DNA sequencer (BioResource Center, Cornell University).

Assessment of colonization density by real-time PCR. PCR primers and probes for *H. pylori* were designed with Primer Express software (Applied
TABLE 1. Sequences of PCR primers and TaqMan probes specific for H. pylori genes, feline GAPDH, and feline cytokines

<table>
<thead>
<tr>
<th>Target</th>
<th>Designation</th>
<th>Sequence</th>
<th>Length of amplicon (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ureB</td>
<td>Hp1-F</td>
<td>GGAATTCGGACGGCTGATTAGCAGAAAGAATGCTAAGAGGTTG</td>
<td>1,700</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Hp2-R</td>
<td>GGAATTCGGACGGCGTATAGCAGAAAGAATGCTAAGAGGTTG</td>
<td>1,200</td>
<td></td>
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<tr>
<td>oipA</td>
<td>oipA-F</td>
<td>TAGAGAGGGTGAGGGGATTC</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td></td>
<td>oipA-R</td>
<td>TGGTGCGGATAATGCCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cagPAI empty site</td>
<td>Luni1</td>
<td>ACTTTTGTGCTAAATAAACGCTG</td>
<td>535</td>
<td>1</td>
</tr>
<tr>
<td>vacAsI s2</td>
<td>VAI-R</td>
<td>ATGGAATAACAAAACACAC</td>
<td>259 (s1)</td>
<td></td>
</tr>
<tr>
<td>vacAsI s2</td>
<td>VAI-F</td>
<td>ATGGAATAACAAAACACAC</td>
<td>286 (s2)</td>
<td></td>
</tr>
<tr>
<td>ureA</td>
<td>ureA-362F</td>
<td>ACAAAGCCAAGAAAAGCTTTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ureA-424R</td>
<td>AGAAATGGAAATGTGAGGCAGAT</td>
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<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAPDH-57F</td>
<td>GCATTGGAATATTGCGT</td>
<td>82</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>GAPDH-138R</td>
<td>GCCCATCAATGACCCCTCAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAPDH-77T</td>
<td>VIC-CTCAACATCATGTCATGCATTTGCTATGAGGTTGCA-TCACGTTAGGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>18S-F</td>
<td>GCCGTTACCACATCCACAGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18S-R</td>
<td>GCTGTAATTACCCCGCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>cat-IFN-g-109F</td>
<td>AAGGGATATTTTTTATGCAAGTAACTCA</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>cat-IFN-g-191R</td>
<td>TCCCTTTCTGAGTTCATTTAATGTT</td>
<td>83</td>
<td></td>
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<tr>
<td>IL-1α</td>
<td>cat-IL-01a-699F</td>
<td>CAAATCGTGGGCCATCAA</td>
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<td></td>
<td>cat-IL-01a-793R</td>
<td>TCTGAAGATCGTAGCAAGGCTTGA</td>
<td>95</td>
<td></td>
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<tr>
<td>IL-1β</td>
<td>cat-IL-01b-415F</td>
<td>CGTTCGCGAACTAACCTACACGA</td>
<td>108</td>
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<tr>
<td>IL-4</td>
<td>cat-IL-04-143F</td>
<td>GCTAGGAGCTGAGGGCTA</td>
<td>81</td>
<td>42</td>
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<tr>
<td>IL-6</td>
<td>cat-IL-06-226F</td>
<td>ATCCCTGTACCCCAACGTAGAA</td>
<td>126</td>
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<tr>
<td>IL-8</td>
<td>cat-IL-08-170F</td>
<td>GGTGCCCAACTCTGAAATC</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>cat-IL-10-330F</td>
<td>CACACCTGACGTCATTTAATG</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>IL-12p40</td>
<td>cat-IL-12p40-253F</td>
<td>TGGCCCTAGTGAGGCTGCTT</td>
<td>81</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>cat-IL-12p40-283T</td>
<td>FAGCMGCGCCCACTGAGGGCT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*VIC, fluorescent reporter dye (ABI Biosystems); FAM, carboxyfluorescein-NHS-ester; TAMRA, carboxytetramethylrhodamine-ester.

Biosystems) and used to amplify DNA from bacterial cultures and gastric tissue samples (Table 1). DNA samples from Helicobacter spp. that are known to colonize the cat stomach, including H. pylori strains CS-1 (feline) and 99QMO3 (feline), and H. felis, H. bizzozeronii, and H. heilmannii (biopsy samples from infected cats), were used to evaluate the specificity of the primers and probes. No signal for ureA was observed with H. heilmannii- and H. bizzozeronii-containing samples. H. felis DNA was detected with this PCR, even though its C_{T} values (a measure of DNA content and amplification efficiency) differed from those of H. pylori by more than 10 cycles when the same amount of DNA per reaction was used. However, as DNA samples from infected and uninfected cats were negative for H. felis DNA by conventional PCR (54), samples were analyzed for ureA by quantitative PCR.

ureA DNA in gastric tissue was quantified with the ABI Prism 7700 sequence detection system (Applied Biosystems). Amplification was carried out in 25-μl reaction volumes that, in addition to 2.5 μl of DNA, contained 1× Taqman buffer A, 5.5 mM MgCl₂, 900 nM each primer, 200 nM probe, 0.05% gelatin (Sigma), 0.01% Tween 20 (Sigma), 200 μM each dATP, dCTP, and dGTP, 400 μM dUTP, 0.025 U of AmpliTaq Gold/μl, and 0.01 U of AmpliEnzyme N-glycosylase/μl. All reagents except primers, probes, gelatin, and Tween 20 were included in the Taqman PCR core kit (Applied Biosystems). Amplification was performed in MicroAmp 96-well plates with a standard amplification protocol recommended by the manufacturer (2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C), and signals were recorded with a charge-coupled device camera controlled by sequence detection software (version 1.6; Applied Biosystems).

Serially diluted DNA from cultured H. pylori strains CS-1 was used to generate a standard curve. The number of H. pylori organisms was calculated from the DNA concentration of the culture by assuming an H. pylori genome size of 1.6 Mbp and that 1 μg of bacterial DNA contains 5.6 × 10^9 organisms. DNA concentrations of cultured H. pylori and each gastric tissue sample were determined by measurement at an optical density at λ of 260 nm. Quantitative values for gastric H. pylori were normalized with the corresponding tissue DNA concentration, and bacterial density was expressed as H. pylori organisms per microgram of gastric DNA.

Cytokine response. Total RNA from biopsy samples or lipopolysaccharide-stimulated macrophages, lymphocytes, and synovial membranes (controls with known cytokine upregulation) was extracted with a RNasy extraction kit according to the manufacturer’s instructions (QIAGEN). After elution of RNA from the QIAGEN spin columns with 55 μl of diethyl pyrocarbonate-treated water, each sample was treated with 1 U of DNase I (amplification grade) following the manufacturer’s protocol (Gibco BRL/Invitrogen, Carlsbad, Calif.). Immediately after this treatment, samples were stored at −80°C. A plasmid containing cDNA for feline IL-4 was a generous gift of Harrie Glansbeek, University of Utrecht, Utrecht, The Netherlands. Reverse transcription reactions were carried out in a GeneAmp 9600 PCR system (Applied Biosystems, Foster City, Calif.). Five microliters of total RNA
carried out in 27.5-l reaction volumes that, in addition to 2.5 μl of a Prism 7700 sequence detection system (Applied Biosystems). Amplification was performed in MicroAmp 96-well plates with a standard amplification protocol recommended by the manufacturer (2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C), and signals were recorded with a charge-coupled device camera controlled by the sequence detection software, version 1.6. Recorded Ct values for feline cytokines were normalized with corresponding Ct values for GAPDH or 18S rRNA, resulting in ΔCt values. ΔCt values were further used to calculate the change in upregulation of cytokines. The average ΔCt value from uninfected cats was subtracted from ΔCt values of the H. pylori-infected cats, resulting in ΔΔCt values that were used to calculate the up- or downregulation (F) of cytokines with the formula $F = 2^{-\Delta\Delta C_T}$ (3).

### TABLE 2. Immunohistochemistry of feline lymphocytes

<table>
<thead>
<tr>
<th>Antigen (reference)</th>
<th>Cell differentiation</th>
<th>Antibody*</th>
<th>Antigen retrieval*</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLA36 (37)</td>
<td>Progenitor B cells</td>
<td>Mouse MAb</td>
<td>MW</td>
<td>1:25</td>
<td>Dako</td>
</tr>
<tr>
<td>B220 antigen of CD45R (35)</td>
<td>Transition of activated lymphocytes to apoptosis</td>
<td>Rat MAb</td>
<td>MW, CSA</td>
<td>1:200</td>
<td>Dako</td>
</tr>
<tr>
<td>CD70a (74)</td>
<td>Reactive B cells</td>
<td>Mouse MAb HM57</td>
<td>MW, CSA</td>
<td>1:200</td>
<td>Dako</td>
</tr>
<tr>
<td>CD3 (71)</td>
<td>T cells</td>
<td>Rabbit PAb</td>
<td>Trypsin</td>
<td>1:200</td>
<td>Dako</td>
</tr>
</tbody>
</table>

* MAb, monoclonal antibody; PAb, polyclonal antibody.

Histology. Serial sections of each block were stained with hematoxylin and eosin and evaluated according to site (cardia, fundus, and pyloric antrum) in a blinded fashion by one pathologist (E.S.) for the degree and type of inflammation, atrophy, and fibrosis. The degree of mononuclear inflammation, fibrosis, and atrophy was graded as +1 (mild), +2 (moderate), or +3 (severe).

The number of lymphoid follicles in each region was counted, and the area of each follicle was measured with digital images (Olympus DP11 microscope digital camera system). Images were analyzed with Image J 1.22d and image analysis programs in the public domain available from the National Institutes of Health.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissues were sectioned at 4 μm and mounted on Probe-on slides (Fisher Scientific, Springfield, N.J.). Immunohistologic staining was performed with streptavidin-biotin-horse-radish peroxidase and a catalyzed signal amplification system according to the manufacturer’s instructions (Dako, Carpinteria, Calif.). The chromogen was 3,3’-diaminobenzidine (Sigma, St. Louis, Mo.), and all slides were lightly counterstained with Gill’s hematoxylin. The specificity, antigen retrieval method, and source of primary antibodies used in this study are summarized in Table 2.

Positive tissue controls consisted of normal feline spleen and lymph node. Negative technique controls were performed by substituting the primary antibody with phosphate-buffered saline, normal rabbit serum, or an isotype-matched irrelevant mouse monoclonal antibody.

Statistical analysis. Differences in inflammation, lymphoid follicles, atrophy, and fibrosis between naturally infected and uninfected cats were evaluated with the Mann-Whitney test. Differences in bacterial colonization density, urease activity, inflammation, lymphoid follicle atrophy, and fibrosis between sites (pylorus versus fundus versus cardia) within each cat were evaluated with the Friedman test. Differences in cytokine expression were evaluated by comparing the ΔCt values of infected and uninfected cats with the Mann-Whitney test. Association between bacterial colonization density, urease activity, inflammation, and lymphoid follicles and the degree of cytokine upregulation was assessed with the Kendall correlation coefficient. Statistical significance was set at $P$ levels of ≤0.05.

**FIG. 1.** Restriction fragment length polymorphism evaluated by HaeIII digestion of ureB PCR products (2). Human H. pylori isolates ATCC 8826, 43504, and 49503 (lanes 1 to 3, respectively); reference strain H. pylori CS1 (lane 4); samples from the H. pylori-infected queen (lane 5) and her three kittens (lanes 6 to 8). Lane M, molecular size markers.
TABLE 3. oipA sequences of PCR amplicons from gastric mucosa from *H. pylori*-infected cats

<table>
<thead>
<tr>
<th>Cat no.</th>
<th>Sequence (5' -&gt; 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATGAAAAGCCCTTCTTAACCTCCTCCCTCTCTACTGTTCTGGCT</td>
</tr>
<tr>
<td>2</td>
<td>ATGAAAAGCCCTTCTTAACCTCCTCCCTCTCTACTGTTCTGGCT</td>
</tr>
<tr>
<td>3</td>
<td>ATGAAAAGCCCTTCTTAACCTCCTCCCTCTCTCTACTGTTCTGGCT</td>
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<tr>
<td>4</td>
<td>ATGAAAAGCCCTTCTTAACCTCCTCCCTCTCTCTACTGTTCTGGCT</td>
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<tr>
<td>5</td>
<td>ATGAAAAGCCCTTCTTAACCTCCTCCCTCTCTCTACTGTTCTGGCT</td>
</tr>
</tbody>
</table>

RESULTS

Infection status. Microscopic evaluation of endoscopically retrieved samples showed that the five cats born into our *H. pylori*-infected colony were infected by 8 to 14 weeks of age with organisms that resembled human *H. pylori*. Gastric biopsy samples taken from these animals exhibited urease activity; *H. pylori*-like S-shaped bacteria were evident in modified Steiner’s stained sections; and PCR tests were positive for *H. pylori*-specific 16S rRNA and ureB gene fragments. HaeIII restriction (restriction fragment length polymorphism) of ureB amplicons showed the same restriction pattern in samples from kittens and their mothers, with bands of approximately 450, 550, and 680 bp in each case (Fig. 1). The pattern was distinct from that of *H. pylori* control strains ATCC 49504, 43504, and 8826 (Fig. 1), as expected, given the great DNA sequence diversity among *H. pylori* strains (2), and indicates that the kittens acquired the infection from their mothers. The mode of transmission was not established. DNA samples from *H. pylori*-infected cats were negative for *H. felis*, *H. heilmannii*, and *H. bizzozeronii* DNA by PCR.

Further endoscopic evaluation 6 months later indicated that *H. pylori* was still present and that the infections were chronic. Necropsy was performed at 8 to 9 months of age, when the cats had been infected for a median duration of 5.5 months (range, 5 to 7 months). Control cats (n = 7) that had not been infected and had been kept in a separate room in the same facility remained free of *H. pylori* (no urease activity; no *H. pylori* detected by PCR or histopathology).

Infected strains were vacA*sl* positive and toxigenic to RK-13 cells (data not shown). They were positive for the cag-PAI empty site, indicating that they completely lacked cagPAI (1). Sequence analysis of oipA amplicons showed the presence of 8 or 10 CT repeats (Table 3), indicating that oipA was in an off rather than an on state (80).

*H. pylori*-like organisms were observed microscopically with Steiner’s stain throughout the stomach, most frequently in the superficial gastric mucus layer and in the lumen of gastric glands, but a small number also seemed to be present within parietal cells. The number of spiral bacteria visible on Steiner-stained sections was higher in the cardia (P < 0.05) than the fundus and the pylorus (Table 4). Further quantitative analysis of infection by PCR and urease activity also indicated that colonization was pangastric, although colonization density was not significantly different between the pylorus, fundus, and cardia (Table 4).

Response to infection. None of the cats in this study showed any clinical signs of overt disease associated with *H. pylori* infection, such as vomiting or listlessness.

Cytokine response mRNA content determined by real-time PCR. The proinflammatory cytokines IFN-γ, IL-1α, IL-1β, and IL-8 were significantly upregulated in *H. pylori*-infected cats (Fig. 2). The degree of upregulation of IFN-γ in infected cats was influenced by gastric site, with the fundus and pylorus showing markedly more upregulation than the cardia. The amount of mRNA for these specific proinflammatory cytokines, which differed significantly from the uninfected controls, increased on average by 17- to 225-fold in the infected cats. For other cytokines, there was a trend for the pylorus and cardia to show more upregulation than the fundus, but this was not statistically significant. Upregulation of the immunomodulatory cytokines IL-4, IL-6, IL-10, and IL-12 was not detected (Fig. 2).

There were significant correlations between IL-1β and IL-8 (P = 0.0003, tau = 0.695), IL-1β and IFN-γ (P = 0.0001, tau = 0.810), and IL-8 and IFN-γ (P = 0.001, tau = 0.619).

Histopathology. Infected cats had more lymphocytic inflammation in the cardia (P < 0.05) than uninfected cats (Table 4). Eosinophils and globular leukocytes were observed solely in infected cats: diffuse eosinophil infiltrates of moderate severity were present in the cardia of three and the fundus of one cat, while globular leukocytes were detected in the pyloric mucosa of two cats. The total number (n) and area (A) of lymphoid aggregates (median [range]) was substantially higher (P > 0.01) in the stomachs of infected cats (n = 31, range, 15 to 50; A = 110.3 mm², range, 53 to 177 mm²). Within the infected cats, fibrosis was more severe in the cardia than the fundus and pylorus (Table 4). There was no significant corre-

TABLE 4. Colonization density and histopathology of kittens with naturally acquired *H. pylori* infection

<table>
<thead>
<tr>
<th>Infection status</th>
<th>Site</th>
<th>Median urease activity score (range)</th>
<th>Median colonization density (range)</th>
<th>Median inflammation score (range)</th>
<th>Median atrophy score (range)</th>
<th>Median fibrosis score (range)</th>
<th>Lymphoid follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>0 (0–2)*</td>
<td>3 (2–3)*</td>
<td>9.6 (0.3–11.4)*</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
<td>5 (0–8)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0 (0–2)</td>
<td>0 (0–1)</td>
<td>10.5 (3.2–64.0)*</td>
<td>0 (0–2)</td>
<td>0 (0–2)</td>
<td>0 (0–2)</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>1 (0–2)*</td>
<td>1 (0–3)*</td>
<td>1.4 (0.4–2.5)*</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
<td>1 (0–1)</td>
</tr>
</tbody>
</table>

a C, cardia; P, pylorus; F, fundus. Activity scores are defined in the text.

b *, P < 0.05; **, P < 0.01 versus uninfected cats.

c #, P < 0.05 versus other gastric sites.
loration between colonization density (evaluated by histology, real-time PCR, or urease activity), gastric inflammation, atrophy, or fibrosis and mucosal cytokine upregulation. There was a significant but weak inverse correlation between IL-1α and the number of lymphoid follicles ($P = 0.03$, tau = $-0.552$).

Among the uninfected specific-pathogen-free cats, there was more inflammation in the pylorus ($P < 0.05$) than the fundus or cardia. There was no effect of gastric site on atrophy in either infected or uninfected cats.

**Immunophenotype.** Lymphoid follicles in the uninfected cats consisted mainly of primary follicles that were positive for BLA.36, a marker for progenitor B cells (Table 5, Fig. 3). The sections were devoid of T cells (CD3^+) and mantle B cells (CD79α^+). Secondary follicles were rarely encountered, but when present, the germinal centers were strongly positive for the B220 antigen of CD45R (a tyrosine phosphatase that enhances signal transduction of the antigen receptor in B and some T cells). In contrast, secondary follicles were frequent in *H. pylori*-infected cats. Germinal centers were strongly positive for BLA.36, while a few lymphocytes in the mantle and in the lamina propria expressed CD79α. Approximately 10% of the follicles were surrounded by a small number of loosely arranged CD3-positive cells, although in rare instances, a dense peripheral band of T cells was detected (Fig. 3). Interestingly, in contrast to uninfected cats, all germinal centers in the *H. pylori*-infected cats were devoid of B220 expression.

**DISCUSSION**

The mechanisms underlying the differences in histopathology and disease pattern between children and adults and the early and late stages of infection are still not well understood (15, 33, 38, 51, 52, 70) and served as a stimulus for our investigation of the early stages of chronic infection. Using real-time reverse transcription-PCR to measure cytokine mRNA expression in cats with naturally acquired, 5- to 7-month-old *H. pylori* infections, we demonstrated upregulation of IFN-γ, IL-1α, IL-1β, and IL-8 with no significant changes in the mRNA content of the immunomodulatory cytokines IL-4, IL-5, and IL-10. These findings are similar to those observed in infected children and adults (5, 30, 44, 45) and suggest that progression along a T_{H}1-type pathway is an early event. We observed a combined upregulation of the cytokines IFN-γ, IL-1β, and IL-8. A correlation between IL-8 and IL-1β levels has also been described in the antral mucosa of infected people (78) and may reflect the direct induction of cytokines and chemokines in epithelial or mononuclear cells by *H. pylori* or its products, the mutual induction and influence of these cytokines, or the initial stimulus of IFN-γ or tumor necrosis factor alpha produced by mononuclear cells (32, 39, 43, 81).

It is notable that despite the upregulation of IL-8, mononuclear cells rather than neutrophils were the predominant infiltrate. A similar lack of association between IL-8 and acute inflammation index has also been observed in *H. pylori*-infected children (45). This may be due to the magnitude of the IL-8 response with a failure to reach the threshold for polymorphonuclear lymphocyte migration. Lymphocytes, in contrast, probably respond to low levels of IL-8 (82) and may facilitate recruitment of macrophages. The presence of predominately mononuclear cells in the gastric mucosa of the infected host could also suggest differences in the immune response during the early and late stages of infection with *H. pylori*.

While in vitro studies indicate that the adherence of bacteria possessing cagPAI and oipA to cultured epithelial cells elicits the production of IL-8 (43, 62, 80), the *H. pylori*-associated factors required for the elaboration of cytokines in vivo have been less clearly defined. The present study demonstrates the in vivo induction of proinflammatory cytokines and chemokines in the face of colonization by *H. pylori* strains that uniformly lacked the entire cagPAI, not just the cagA marker, and did not express functional oipA. Our findings are consistent with those in people, where there is a broad overlap in the levels of chemokines and cytokines observed in the antral biopsy samples of adults that were infected with cagA-positive or -negative strains (57, 63, 78) or with cagPAI-positive and -negative strains that contained either active or inactive oipA (77). In particular, 43% of 60 biopsy specimens with cagPAI^+, oipA^on *H. pylori* strains had IL-8 levels similar to those of samples that harbored *H. pylori* strains with no cagPAI and oipA in the off position (77), making it difficult to reliably predict the clinical outcome in infected individuals with different strains of *H. pylori* (28, 36).

As up to 50 pg of IL-8/mg is present in the antral biopsy samples of people colonized by cagPAI^-^oipA-off *H. pylori* strains (77), other bacterial factors are also involved in the development of the inflammatory response. Probably genes encoded by alleles of other putative virulence-associated genes, e.g., vacuolating cytotoxin (vacA) or iceA (the *H. pylori* strains in the studied cats were vacA s1m2 IceA1 [unpublished observations]), urease (32), or ammonia production, are key players in the induction of proinflammatory cytokines.

Bacterial colonization density may also be a factor in the mucosal inflammatory response against *H. pylori* and has been

<table>
<thead>
<tr>
<th>Cats</th>
<th>BLA.36</th>
<th>B220/CD45RA</th>
<th>CD79α^+</th>
<th>CD3^+</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. pylori</em> infected</td>
<td>2+</td>
<td>2+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Uninfected</td>
<td>3+</td>
<td>0</td>
<td>1+</td>
<td>1+</td>
</tr>
</tbody>
</table>

* Scores: 0, no lymphocytes stained; 1+, up to 25% of lymphocytes stained; 2+, 25 to 50% of lymphocytes stained; 3+, 50 to 100% of lymphocytes stained.
<table>
<thead>
<tr>
<th>Uninfected cats</th>
<th>H. pylori-infected cats</th>
</tr>
</thead>
<tbody>
<tr>
<td>H&amp;E</td>
<td></td>
</tr>
<tr>
<td>BLA.36</td>
<td></td>
</tr>
<tr>
<td>B220</td>
<td></td>
</tr>
<tr>
<td>CD79α</td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td></td>
</tr>
</tbody>
</table>
correlated with IL-8 levels in the antrum of patients with cagA+ and oipA-on strains of *H. pylori* (63, 77, 79). To test this relationship, we rigorously assessed colonization density by real-time PCR directed against *H. pylori* ureA, examination of Steiner-stained sections, and semiquantitative evaluation of urease activity. While the presence or absence of organisms or urease activity was correlated with cytokine upregulation, we found no correlation between the degree of colonization or urease activity and the cytokine response in cats colonized with cagPAI- oipA-off strains.

One of the most striking findings of the present study was the degree of lymphoid follicular hyperplasia in the fundus and antrum of *H. pylori*-infected cats. These findings are consistent with previous studies in people and animals infected with *H. pylori*, *H. heilmannii*, and *H. felis* and suggest that lymphoid hyperplasia is a response against infection with *Helicobacter* spp. in general (26, 40). The lymphoid aggregates in *H. pylori*-infected cats in the present study consisted primarily of B cells (BLA36+/CD3- cells) with relatively few T cells (CD3+ cells) found at the periphery of an occasional follicle. These results differ from those reported by Fox et al., who demonstrated large aggregates of T cells surrounding mucosal follicles in infected cats (24). Additionally, in the present study, B cells in follicles from uninfected cats strongly expressed the B220 antigen of CD45R and were negative for CD79a, while those of infected cats were uniformly negative for B220 and weakly expressed CD79a. This is in contrast with the results of a previous study of *H. pylori*-infected cats that showed strong staining of follicles for CD45-RA (20).

These interstudy differences may have arisen because of differences in the age of the cats and the duration of infection, as the cats in the present study were under 1 year of age and had been infected for 5 to 7 months, whereas cats in previous studies were mature adults up to 5 to 6 years old (20, 24). This possibility is supported by studies in *H. pylori*-infected dogs, in whom the number of T cells and follicles grew as the duration of infection increased (60). However, differences in methodology may also account for some discrepancies, as the antigen retrieval techniques and antibodies differed between studies. The antibodies employed in the present study have been validated for use in cats (see Table 2) and are used in the clinical setting for immunohistochemistry. It is unlikely that variation in *H. pylori* strains was responsible for differences in immunophenotype, as the *H. pylori*-infected colony reported here was derived from the one reported by Fox et al. (24), and bacterial strains isolated from cats appear uniform in global genotype (i.e., cagPAI+, vacAs1/m2, same restriction fragment length polymorphism pattern).

While the factors driving the B-cell hyperplasia remain to be fully elucidated, *Helicobacter*-derived factors such as urease stimulate cloned T cells to proliferate and provide B-cell help (13). Stimulated cloned T cells frequently produce IFN-γ as the predominant cytokine (13, 14), and this can promote B-cell follicle and germinal center formation (47). The dominant intratumoral T-cell population in people with early low-grade mucosa-associated lymphoid tissue lymphoma also produces IFN-γ (59). Given the close association of IFN-γ and B-cell follicles, it is noteworthy that in the present study both lymphoid follicle hyperplasia and upregulation of IFN-γ were absent from the cardia but present in the fundus and antrum.

The strong upregulation of IFN-γ in *H. pylori*-infected people, cats, and monkeys (50) and minimal pathological changes in *H. pylori*-infected IFN-γ knockout mice (61) suggest that *H. pylori* infection is dominated by a Th1-type response. However, B-cell hyperplasia and progression to MALT lymphoma are thought to require Th2-type responses: inbred BALB/c (Th2 biased) but not C57BL/6 (Th1 biased) (34) mice develop lymphoid hyperplasia and MALT-lymphoma (19), and low-grade B-cell lymphomas require Th2-type cytokine for in vitro growth and differentiation (29). Thus, the activation of both the Th1 and Th2 pathways is likely required to create the mixture of lymphoid follicle hyperplasia and a proinflammatory Th1-type mucosal response observed in people and outbred animals such as nonhuman primates, Swiss Webster mice, and cats (21, 50). Differences in Th1/Th2 balance may help to explain the apparent progression of the predominantly lymphofollicular inflammation in children to the neutrophil-dominated inflammation observed in adults, but this has not been studied in detail.

It is noteworthy that lymphoma accounts for 26 to 33% of malignant tumors in cats and alimentary lymphoma is the most common anatomic form (48, 71). Whether infection with *Helicobacter* spp. has a role in the development of MALT lymphoma in cats, as it does in people, remains to be determined. However, the decrease in the expression of B220, an epitope of the CD45 family of glycoproteins (53) that is thought to play a role in the transition of activated lymphocytes to apoptosis (11) by B cells in lymphoid follicles in *H. pylori*-infected cats, suggests that these B cells are proliferating out of control of immune homeostatic mechanisms.

In addition to impacting gastric morphology, cytokines may also affect gastric function (8–10, 41). Cats infected with *H. pylori* ss1 (a nominally cagPAI-positive but functionally cagPAI-inactive nontoxicogenic [vacAs2] strain [73]) exhibit transient hypochlorhydria (65), which is analogous to the transient hypochlorhydria reported in the early stages of infection in people (31). Thus, in vivo investigation of the interplay between inflammatory mediators, bacterial products, and gastric function may be facilitated by the cat model of infection.

In summary, we found that a proinflammatory response characterized by upregulation of IFN-γ, IL-1α, IL-1β, and IL-8 and marked lymphoid hyperplasia develops early in the course of *H. pylori* infection in cats. This response was independent of the presence of the bacterial virulence factors cagPAI and...
The inflammatory and immune responses observed in young infected cats appear to be more similar to those reported in infected children than in adults and suggest that the cat model will be useful for the in vivo elucidation of host–Helicobacter pylori interactions during the early stages of infection.

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