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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 gamma interferon (IFN- $\gamma$ ), interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , 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 *vacAs1* but lacked *cagPAI* and an active *oipA* gene. Colonization density was uniform throughout the stomach. Upregulation of IFN- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , 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 BLA.36 and B220 but negative for CD3 and CD79 $\alpha$ , whereas in infected cats they were frequent and positive for BLA.36, CD79 $\alpha$ , and CD3 but negative for B220. Upregulation of IFN- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , 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 atrophy 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 inflammatory and immune response to infection appears to be pivotal for the development of disease (12, 49, 55, 56). Chronic infection of adults with *H. pylori* is characterized by the infiltration 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 inter-

feron (IFN- $\gamma$ ) rather than IL-4 or IL-5, indicating a strong bias toward a T<sub>H</sub>-1 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-1 $\alpha$ , IL-1 $\beta$ , 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- $\kappa$ B and IL-8 secretion in vitro (43, 62). Thus, it has become widely accepted that IL-8 induction in response to colonization with *cagPAI*<sup>+</sup> *H. pylori* is a key step in the pathogenesis of neutrophilic gastritis observed in adults with *H. pylori* infection. However, *cagPAI*<sup>+</sup> 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

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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  $T_H1$ -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 *cagPAI*, *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 (see "Endoscopic biopsy" below) and had been infected for a median duration of 5.5 months (range, 5 to 7 months). Seven domestic shorthair specific-pathogen-free, *Helicobacter*-free cats (five females, 7 months old, and two males, 5 months old) housed in a different room in the same building served as an uninfected control group.

The presence or absence of gastric *Helicobacter* spp. was ascertained in all cats by evaluating gastric biopsy samples for urease activity, tissue sections for the presence of *Helicobacter*-like organisms (HLO), and gastric biopsy samples for *Helicobacter* DNA (see "Gastric biopsy" below). All cats were housed in a barrier facility with a controlled environment (lighting, 12 h on, 12 h off; temperature, 20 to 21°C; humidity, 35 to 45%) at Cornell University, were fed a standard commercial diet (Teklad lab cat diet) ad libitum and had constant access to water throughout the study. Cornell University operates under an approved Animal Welfare Assurance (A3347-01, A-3125-01) and is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The Institutional Animal Care and Use committees at Cornell University approved the project.

**Endoscopic biopsy.** Endoscopic biopsy samples of the stomach were obtained from each cat with a video gastroscope and biopsy forceps (Fujinon EG7-FP2; 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 colony between 8 and 14 weeks of age and in specific-pathogen-free cats aged 8 to 10 weeks a week after entering the facility. Biopsies were obtained from the pyloric antrum (incisura to pyloric sphincter), the fundus (greater curvature), and the cardia. Two biopsy samples were taken from each site for urease testing, three from each site for histopathology, and one from each site for PCR. Samples for PCR were frozen at  $-80^{\circ}\text{C}$  pending analysis. The endoscope and the biopsy forceps were 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.** Full-thickness gastric tissue 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 urease activity and *Helicobacter* DNA by PCR. A further two samples were obtained for cytokine analysis, snap frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  pending analysis. A full-thickness strip of stomach along the greater curvature, cut from the cardia to pylorus, rolled with the pylorus innermost and secured with a needle (20 gauge), was fixed in 10% buffered formalin for 24 h and used for the evaluation of histopathology, histomorphometry, and immunophenotyping of lymphocytes.

**Urease activity.** Urease activity of gastric tissue was evaluated as previously described (58). In short, gastric mucosal biopsy samples were placed in sterile tubes containing 200  $\mu\text{l}$  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  $\mu\text{m}$ . 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  $-80^{\circ}\text{C}$ . 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 desiccator, and resuspended with sterile Tris-EDTA buffer. The concentrated PCR product (10  $\mu\text{l}$ ) was digested with 10 U of the restriction enzyme *HaeIII* in a buffer for 2 h at  $37^{\circ}\text{C}$  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 *cagA*, *cagPAI* (with primers directed against the *cag* empty site [1]), *oipA*, and *vacA* allele (Table 1) was carried out with 1  $\mu\text{l}$  of extracted DNA in 50- $\mu\text{l}$  volumes of Qiagen mastermix (Qiagen) and 500 nM primer concentration (see Table 1 for primer sequences) for 30 (*cagA* and *vacA*) or 40 (*oipA*) cycles under the following conditions: 2 min at  $94^{\circ}\text{C}$ , 2 min at  $55^{\circ}\text{C}$  ( $52^{\circ}\text{C}$  for *oipA*), and  $72^{\circ}\text{C}$  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 ureA* 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

Target	Designation	Sequence <sup>a</sup> (5' → 3')	Length of amplicon (bp)	Reference
<i>ureB</i>	Hp1-F	GGAATTCCAGATCTATGAAAAAGATTAGCAGAAAAG	1,700	54
	Hp2-R	GGAATTCGTCGACCTAGAAAATGCTAAAGAGTTG		
<i>oipA</i>	oipA-F	TAGAGAGGTGAGCGGATTG	1,200	
	oipA-R	TGGTTGCGATAAATGCCCG		
<i>cagPAI</i> empty site	Luni1	ACATTTTGGCTAAATAAACGCTG	535	1
	R5280	GGTTGCACGCATTTTCCCTTAATC		
<i>vacAs1 s2</i>	VA1-F	ATGGAAATACAACAAACACAC	259 (s1)	
	VA1-R	CTGCTTGAATGCGCCAAAC	286 (s2)	
<i>ureA</i>	ureA-362F	ACGAAGGCCAAAAAGCGTTA	81	
	ureA-442R	AGAAATGGAAGTGTGAGCCGAT		
	ureA-394T	FAM-AAAAATGTTGGCGACAGACCGGTTCA-TAMRA		
GAPDH	GAPDH-57F	GCCGTGGAATTTGCGCT		
	GAPDH-138R	GCCATCAATGACCCCTTCAT	82	42
	GAPDH-77T	VIC-CTCAACATCATGGTCATCATGTTCCAGTATGATTCCA-TAMRA		
18S rRNA	18S-F	CGGCTACCACATCCAAGGAA		
	18S-R	GCTGGAATTACCGCGCT	~186	4
	18S-T	VIC-TGCTGGCACCAGACTTGCCCTC-TAMRA		
IFN- $\gamma$	cat-IFN-g-109F	AAGGGATATTTTAAATGCAAGTAATCCA		
	cat-IFN-g-191R	TCCTCTTTCCAGTTCTTCAAATGT	83	
	cat-IFN-g-138T	FAM-TGTAGCAGATGGTGGTTCGCTTTTCGT-TAMRA		
IL-1 $\alpha$	cat-IL-01a-699F	CAAATCAGTTGCCCATCCAAA		
	cat-IL-01a-793R	TCTGAAAGTCAGTGACAGAGGGTAGT	95	
	cat-IL-01a-724T	FAM-TTCATTGCCACACAGGAAGAACAACCTGGT-TAMRA		
IL-1 $\beta$	cat-IL-01b-415F	CGTCCGAGACATAAGCCAAA		
	cat-IL-01b-522R	TCATGCGGAACACCACCTTGTT	108	
	cat-IL-01b-438T	FAM-AGCCTGGTGTGTCTGGCTCATACGA-TAMRA		
IL-4	cat-IL-04-143F	GCATGGAGCTGACCGTCAT		
	cat-IL-04-223R	CGGTGTGGCTCTGCAGA	81	42
	cat-IL-04-169T	FAM-TGGCAGCCCCCTAAGAACACAAGTGACAA-TAMRA		
IL-6	cat-IL-06-226F	ATCCCTGTCCCAACCGTAGAA		
	cat-IL-06-351R	CCTGAGGCTGAACGAAGGAA	126	
	cat-IL-06-283T	FAM-TGGCTGAGGCACACAACATTCACCT-TAMRA		
IL-8	cat-IL-08-170F	GTGGCCCACTGTGAAACT		
	cat-IL-08-267R	CACAACCTTCTGCACCCACTT	98	
	cat-IL-08-224T	FAM-AGGTGTGCCTGGACCCCAAGCA-TAMRA		
IL-10	cat-IL-10-330F	CTCCCTGGGAGAAAAGCTGAA		
	cat-IL-10-419R	TCCACCACCTTGCTCTTGT	90	
	cat-IL-10-364T	FAM-AGACTGCGGCGCTGTCATCGATTT-TAMRA		
IL-12p40	cat-IL-12p40-253F	TGGCTTCAGTTGCAGGTTCTT		
	cat-IL-12p40-333R	TGGACGCTATTACAAGCTCA	81	42
	cat-IL-12p40-283T	FAM-CGGTTTGATGATGTCCCTGATGAAGAAGCT-TAMRA		

<sup>a</sup> VIC, fluorescent reporter dye (ABI Biosystems); FAM, carboxyfluorescein-NHS-ester; TAMRA, carboxytetramethylrhodamine-NHS-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 98QM03 (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 27.5- $\mu$ l reaction volumes that, in addition to 2.5  $\mu$ l of DNA and water, contained 1 $\times$  Taqman buffer A, 5.5 mM MgCl<sub>2</sub>, 900 nM each primer, 200 nM probe, 0.05% gelatin (Sigma), 0.01% Tween 20 (Sigma), 200  $\mu$ M each dATP, dCTP, and dGTP, 400  $\mu$ M dUTP, 0.025 U of AmpliTaq Gold/ $\mu$ l, and 0.01 U of AmpErase N-glycosylase/ $\mu$ l. 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* 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  $\mu$ g of bacterial DNA contains  $5.6 \times 10^8$  organisms. DNA concentrations of cultured *H. pylori* and each gastric tissue sample were determined by measurement at an optical density at  $\lambda$  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 an RNeasy extraction kit according to the manufacturer's instructions (Qiagen). After elution of RNA from the Qiagen spin columns with 55  $\mu$ l 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



TABLE 2. Immunohistochemistry of feline lymphocytes

Antigen (reference)	Cell differentiation	Antibody <sup>a</sup>	Antigen retrieval <sup>b</sup>	Dilution	Source
BLA.36 (37)	Progenitor B cells	Mouse MAb	MW	1:25	Dako
B220 antigen of CD45R (35)	Transition of activated lymphocytes to apoptosis	Rat MAb	MW, CSA	1:200	Serotec
CD79α (74)	Reactive B cells	Mouse MAb HM57	MW, CSA	1:200	Dako
CD3 (71)	T cells	Rabbit PAB	Trypsin	1:200	Dako

<sup>a</sup> MAb, monoclonal antibody; PAB, polyclonal antibody.  
<sup>b</sup> MW, microwave; CSA, catalyzed signal amplification (Dako).

(approximately 0.5 μg of total RNA) in 55-μl reaction volumes consisting of 1× PCR buffer II, 5.5 mM MgCl<sub>2</sub>, 500 μM concentration of each of the four deoxynucleoside triphosphates (all from Applied Biosystems), 0.4 U of RNasin RNase inhibitor/μl (Promega, Madison, Wis.), 5.0 U of Moloney murine leukemia virus reverse transcriptase/μl (Amersham Life Science, Piscataway, N.J.), and 2.5 μM random hexamers (Applied Biosystems) was transcribed into cDNA at 25°C for 10 min, followed by 48°C for 30 min, and at 95°C for 5 min.

PCR primers and probes for feline gamma interferon (IFN-γ), IL-1α, IL-1β, IL-6, IL-8, and IL-10 were designed with Primer Express software (Applied Biosystems) and used to amplify their respective cDNAs (Table 1). Published primers and probes designed to amplify IL-4, IL-12 p40, and a fragment from the feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (42) and a universal 18S rRNA detection kit (Applied Biosystems [4]) were used to monitor the amount of RNA present in the reaction. cDNA was quantified with the ABI Prism 7700 sequence detection system (Applied Biosystems). Amplification was carried out in 27.5-μl reaction volumes that, in addition to 2.5 μl of cDNA solution from the reverse transcription reaction and water, contained 1× Taqman buffer A, 5.5 mM MgCl<sub>2</sub>, 900 nM each primer, 200 nM probe, 0.05% gelatin (Sigma), 0.01% Tween 20 (Sigma), 290 μM each dATP, dCTP, and dGTP (including transfer from reverse transcription), 400 μM dUTP, 0.025 U of AmpliTaq Gold/μl, and 0.01 U of AmpErase 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 the sequence detection software, version 1.6. Recorded *C<sub>T</sub>* values for feline cytokines were normalized with corresponding *C<sub>T</sub>* values for GAPDH or 18S rRNA, resulting in values termed Δ*C<sub>T</sub>*. Δ*C<sub>T</sub>* values were further used to calculate the change in upregulation of cytokines. The average Δ*C<sub>T</sub>* value from uninfected cats was subtracted from Δ*C<sub>T</sub>* values of the *H. pylori*-infected cats, resulting in ΔΔ*C<sub>T</sub>* values that were used to calculate the up- or downregulation (*F*) of cytokines with the formula  $F = 2^{-\Delta\Delta C_T}$  (3).

**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-horseradish 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 Δ*C<sub>T</sub>* 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.

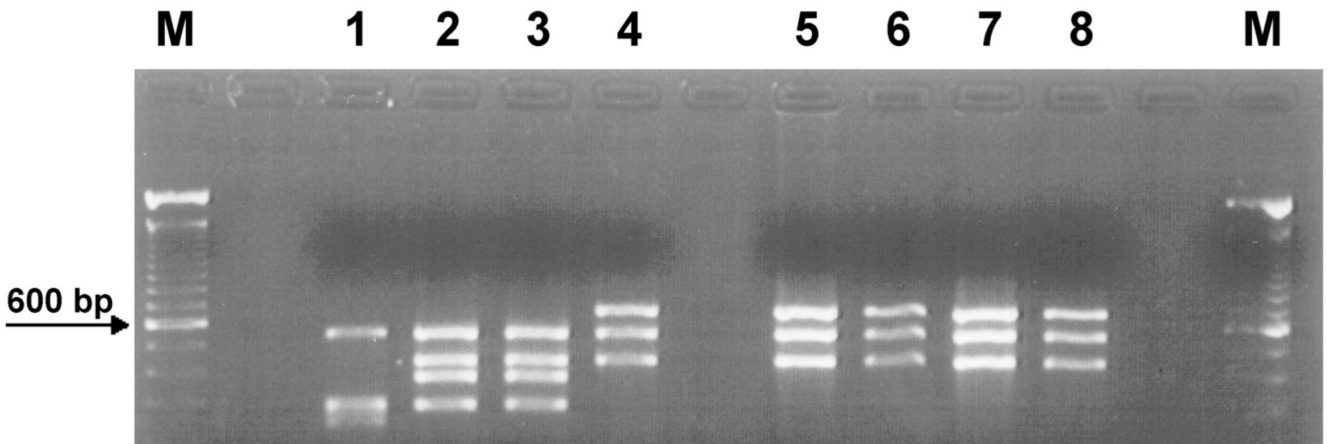
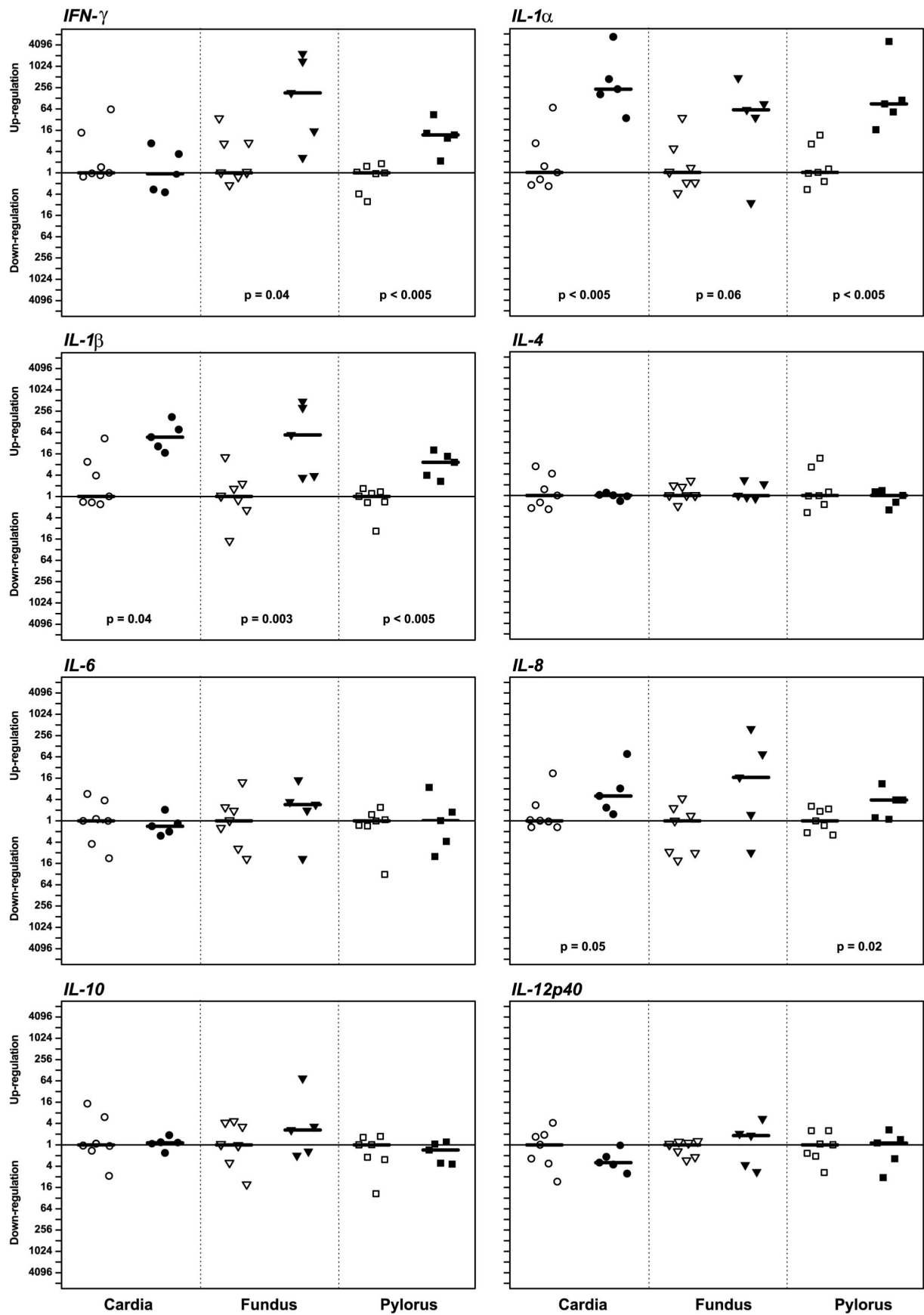


FIG. 1. Restriction fragment length polymorphism evaluated by *Hae*III digestion of *ureB* PCR products (2). Human *H. pylori* isolates ATCC 8826, 43504, and 49503 (lanes 1 to 3, respectively); reference strain *H. pylori* CS1 (lane 4); samples from the *H. pylori*-infected queen (lane 5) and her three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







lation 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-1 $\alpha$  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 (CD79 $\alpha^{+}$ ). 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 CD79 $\alpha$ . 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- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , 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- $\gamma$ , IL-1 $\beta$ , and IL-8. A correlation between IL-8 and IL-1 $\beta$  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- $\gamma$  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 infil-

TABLE 5. Gastric lymphocyte population by immunohistochemical staining<sup>a</sup>

Cats	BLA.36	B220/CD45RA	CD79 $\alpha$	CD3
Uninfected	2+	2+	0	0
<i>H. pylori</i> infected	3+	0	1+	1+

<sup>a</sup> 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.

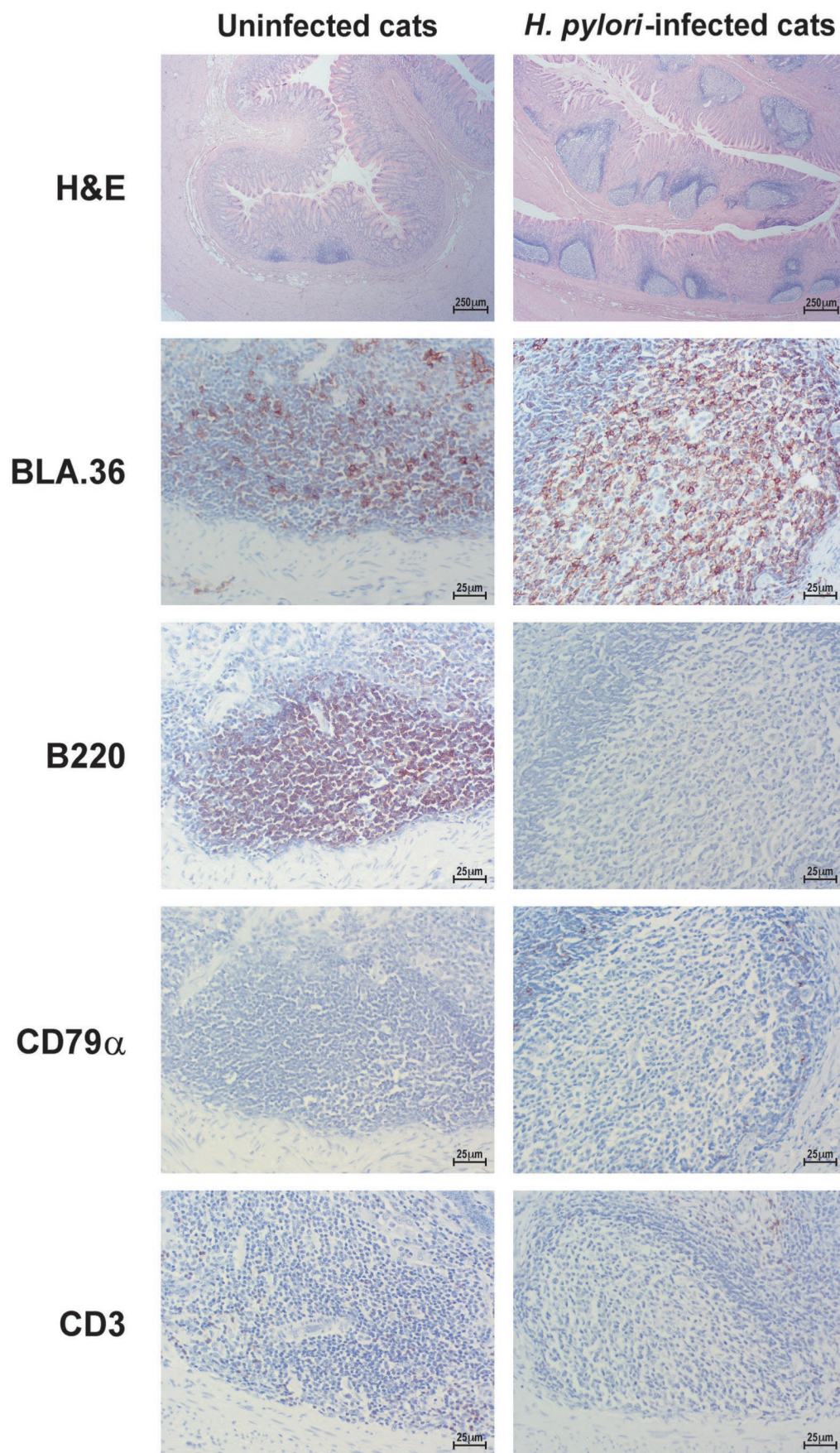
trate. A similar lack of association between IL-8 and acute inflammation index has also been observed in *H. pylori*-infected children (45). This may be due to the magnitude of the IL-8 response with a failure to reach the threshold for polymorphonuclear lymphocyte migration. Lymphocytes, in contrast, 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

FIG. 2. Cytokine mRNA expression in individual tissue samples taken from the cardia, fundus, and pylorus in seven uninfected and five *H. pylori*-infected cats 5 months after infection. Cytokine mRNA was detected by real-time PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA measured in the same specimen. Constitutive cytokine mRNA levels were detected in uninfected healthy cats. Thus, values in infected cats were expressed as upregulation or downregulation above or below the baseline. Values for uninfected and *H. pylori*-infected cats are presented for individual samples from all cats, and median values of each group are shown as a bar.





correlated with IL-8 levels in the antrum of patients with *cagA*<sup>+</sup> 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*<sup>-</sup> *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<sup>+</sup>) with relatively few T cells (CD3<sup>+</sup> 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 CD79 $\alpha$ , while those of infected cats were uniformly negative for B220 and weakly expressed CD79 $\alpha$ . 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 immunophenotyping. It is unlikely that variation in infecting *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*<sup>-</sup>, *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- $\gamma$  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- $\gamma$  (59). Given the close association of IFN- $\gamma$  and B-cell follicles, it is noteworthy that in the present study both lymphoid follicle hyperplasia and upregulation of IFN- $\gamma$  were absent from the cardia but present in the fundus and antrum.

The strong upregulation of IFN- $\gamma$  in *H. pylori*-infected people, cats, and monkeys (50) and minimal pathological changes in *H. pylori*-infected IFN- $\gamma$  knockout mice (61) suggest that *H. pylori* infection is dominated by a T<sub>H</sub>-1 type response. However, B-cell hyperplasia and progression to MALT lymphoma are thought to require T<sub>H</sub>-2 type responses: inbred BALB/c (T<sub>H</sub>-2 biased) but not C57BL/6 (T<sub>H</sub>-1 biased) (34) mice develop lymphoid hyperplasia and MALT-lymphoma (19), and low-grade B-cell lymphomas require T<sub>H</sub>-2-type cytokine for in vitro growth and differentiation (29). Thus, the activation of both the T<sub>H</sub>-1 and T<sub>H</sub>-2 paths is likely required to create the mixture of lymphoid follicle hyperplasia and a proinflammatory T<sub>H</sub>-1 type mucosal response observed in people and outbred animals such as nonhuman primates, Swiss Webster mice, and cats (21, 50). Differences in T<sub>H</sub>-1/T<sub>H</sub>-2 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 nontoxigenic [*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- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , 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

FIG. 3. Sections from gastric tissue samples were stained with hematoxylin and eosin or immunohistochemically. Specimens from uninfected cats contained rare secondary lymphoid follicles that reacted strongly with BLA.36 (a marker for progenitor B cells) and the B220 antigen of CD45R (a tyrosine phosphatase that enhances the signal transduction of the antigen receptor in B and some T cells). The follicles were devoid of CD79 $\alpha$ -positive (a marker for reactive B cells) and CD3-positive T cells. Gastric tissue in *H. pylori*-infected cats contained multiple secondary lymphoid follicles that stained heavily with BLA.36 and slightly with CD79 $\alpha$  and CD3. No B220-positive cells were detected in these follicles.

*oipA*. 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-*H. pylori* interactions during the early stages of infection.

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