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**Helicobacter pylori-Specific Antibodies Impair the Development of Gastritis, Facilitate Bacterial Colonization, and Counteract Resistance against Infection**

Ali A. Akhiani, Karin Schön, Lennart E. Franzén, Jacques Pappo, and Nils Lycke

In recent years, Abs have been considered a correlate rather than an effector of resistance against *Helicobacter pylori* infection. However, it is still poorly understood to what extent Ab production correlates with gastric immunopathology. Here we report that Abs not only are dispensable for protection, but they are detrimental to elimination of the bacteria and appear to impair gastric inflammatory responses. We found that the initial colonization with *H. pylori* bacteria was normal in the B cell-deficient (µMT) mice, whereas at later times (>8 wk) most of the bacteria were cleared, concomitant with the development of severe gastritis. In contrast, wild-type (WT) mice exhibited extensive bacterial colonization and only mild gastric inflammation, even at 16 wk after inoculation. Oral immunizations with *H. pylori* lysate and cholera toxin adjuvant stimulated comparable levels of protection in µMT and WT mice. The level of protection in both strains correlated well with the severity of the postimmunization gastritis.

Thus, T cells were responsible for the gastritis, whereas Abs, including potentially host cell cross-reactive Abs, were not involved in causing the gastritis. The T cells in µMT and WT mice produced high and comparable levels of IFN-γ to recall Ag at 2 and after 8 wk, whereas IL-4 was detected after 8 wk only, indicating that Th1 activity dominated the early phase of protection, whereas later a mixed Th1 and Th2 activity was seen.

The hallmarks of infection with *Helicobacter pylori* bacteria are the chronicity despite the existence of strong specific local and systemic humoral and cell-mediated immunity (1, 2). Although resistance against *H. pylori* infection has been extensively studied in patients as well as in the different animal models, we still lack a basic understanding of the mechanisms responsible for bacterial evasion of host protection and of which factors drive the immunopathology associated with infection (1–3). *H. pylori* is the principal cause of chronic gastritis, peptic ulcers, gastric adenocarcinomas, and lymphomas in humans (4–6).

Whether the type of immune responses elicited by *H. pylori* organisms contribute to the immunopathology or just fail to protect against persistence of the infection is incompletely known. For example, are some Ags more involved with immunopathology whereas others elicit protective immunity?

Using genetically modified mice and active immunization with *H. pylori* Ags and adjuvant, it has become clear that CD4⁺ T cells are a major component of host resistance against infection, whereas Abs appear to play a subordinate role in protection (1). Thus, it was found that MHC class II-deficient mice failed to develop protection, whereas B cell-deficient (µMT) mice were fully protected after immunization (7–10). However, the precise role of Abs in resistance against *H. pylori* infection has not been adequately evaluated in previous studies. In patients, *H. pylori* infection induces autoantibodies reactive with gastric epithelial cells, which could drive gastritis (11). Also, mice that bear hybridomas secreting an *H. pylori* cross-reactive mAb demonstrated a gastritis-like disease (12). Moreover, the postimmunization gastritis, seen in well-protected mice, is most often associated with a strong local Ab response in the gastric mucosa (13–16). Abs could be directly cytolytic to epithelial cells through activation of complement, inducing apoptosis or triggering an Ab-dependent cellular cytotoxicity reaction, and in this capacity they could be involved in the *H. pylori*-induced gastritis (17–21). Therefore, specific Ab production may be detrimental to host resistance against *H. pylori* infection.

The severity of the postimmunization gastritis depends on the mouse strain as well as the *H. pylori* bacterial strain (22–24). Because severe postimmunization gastritis characteristically has been demonstrated only in well-protected mice, it has raised the question of whether protection is in fact the result of aggravated inflammation, making the gastric mucosa inhospitable to bacterial colonization (13–16). Data from infected patients as well as from mice challenged with *H. pylori* exhibit strong T cell infiltration with a typical Th1 phenotype in the gastric mucosa (25–27). Both well-protected and chronically infected mice demonstrate a Th1 dominance, and IFN-γ and IL-12 have been found to play major roles in driving *Helicobacter*-induced gastritis as well as being responsible for protection (16, 27–31). However, at variance with the notion that postimmunization gastritis is necessary for protection, Garhart et al. (31) recently reported that immunized IFN-γ and IL-12-deficient mice exhibited comparable inflammation to that of wild-type (WT) mice, but IL-12-deficient mice failed to develop protection, whereas IFN-γ-deficient mice did. This group has previously also shown that postimmunization gastritis subsides over time, and eventually immunized challenged mice were found to be culture negative and to have normal histology (32). In support of their findings, we demonstrated in IL-4-deficient mice that postimmunization gastritis was significantly milder than in WT mice, whereas later a mixed Th1 and Th2 activity was seen.

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3 Abbreviations used in this paper: µMT, B cell deficient; WT, wild type; CT, cholera toxin; Ly, lysate; AP, alkaline phosphatase; NS, not significant.
mice, despite a similar level of protection (30). However, we do not know whether local Ab production differed among the mouse strains, but most certainly there were differences in the production and quality of local Abs among IFN-γ-, IL-12-, and IL-4-deficient mice that could have affected the protection and immunopathology differently (29, 33, 34).

In humans and mice, H. pylori infection stimulates strong specific IgG and IgA Ab production in serum and in the gastric mucosa (35–38). Local IgA and in some studies local IgG Ab production have been implicated in protection, but firm evidence for such action is largely lacking (8, 39–42). By passive transfer of serum from immunized mice to naive recipient mice, Ermak et al. (8) showed that specific circulating Abs failed to protect against infection. In contrast, a recent study by Keenan et al. (42) showed that H. pylori-specific IgG rather than IgA correlated with protection in WT mice immunized intranasally with H. pylori outer membrane vesicles and cholera toxin (CT) adjuvant. Studies in IgA-deficient mice clearly ruled out local neutralizing IgA Abs as being important for protection (9). However, specific IgG or IgM Abs also were not required for protection after immunization in μMT mice, which were protected to the same extent as WT mice (8–10, 43, 44). Moreover, μMT mice exhibited a similar degree of gastric pathology, characterized by a mixed infiltration of activated lymphocytes, macrophages, and neutrophils, but the studies presented conflicting findings about the correlation between gastritis and the level of colonizing H. pylori bacteria (8, 9, 44, 45).

The present study was undertaken to investigate whether specific Abs affect the immunopathology seen in response to infection with H. pylori bacteria and how Abs may influence the development of gastric inflammation and bacterial colonization. Special focus was given to the postimmunization gastritis phenomenon as in μMT and WT mice. Understanding the role of Abs in host resistance against H. pylori infection could be of vital importance for the development of a vaccine that stimulates strong protection with less risk of ensuing immunopathology.

Materials and Methods

Mice

B cell-deficient mice (46) backcrossed for eight generations onto C57BL/6 mice were bred in ventilated cages under pathogen-free conditions at the animal facility at the Department of Medical Microbiology and Immunology, Göteborg University (Göteborg, Sweden). WT C57BL/6 mice were obtained from B & K Universal (Sollentuna, Sweden). All mice were sex-matched and quality of local Abs among IFN-γ-, IL-12-, and IL-4-depleted mice were used throughout the study.

Immunization

Groups of 10 mice were immunized orally with a blunt feeding needle (Popper & Sons, New Hyde Park, NY) four times at weekly intervals with 500 μg of H. pylori whole-cell lysate (Ly) Ags and 10 μg of CT Ag of C. The tissues were placed into Histocon (Histolab Products) at 4°C until they were used.

H. pylori growth conditions and challenge

H. pylori SS1 (Sydney strain) was grown on tryptic soy agar plates (BD Biosciences, Cockeysville, MD) containing 5% sheep blood and 100 μg of vancomycin, 3.3 μg of polymyxin B, 200 μg of bacitracin, 10.7 μg of nalidixic acid, and 30 μg of amphotericin B (Sigma-Aldrich, St. Louis, MO) per ml. The plates were incubated for 48 h at 37°C under microaerobic conditions (10% CO2, 5% O2, and 85% N2). The bacteria were then harvested and inoculated into bruccella broth (BD Biosciences) supplemented with 5% heat-inactivated FCS (Biochrom, Berlin, Germany). A gas mixture consisting of 5% O2, 10% CO2, and 85% N2 was injected directly into culture flasks containing the bacteria before they were sealed and shaken at 120 rpm at 37°C in an incubator for 24 h. Cultures were grown to an OD550 of 0.3 (~5 × 108 CFU/ml) and diluted in bruccella broth for inoculation. Before use, H. pylori cells were analyzed in wet mounts to assess motility and morphology and were subjected to urease, catalase, and oxidase tests. To establish a primary H. pylori infection, mice were inoculated intragastrically via a 20-gauge feeding needle with a 500-μl suspension of H. pylori containing 3 × 107 CFU of the bacteria on two consecutive days and were sacrificed at 2, 8, or 16 wk postinoculation. To assess protection after immunization, the mice were challenged with 5 × 106 CFU of H. pylori 2 wk after the last immunization. At 2, 8, or 18 wk postchallenge, mice were sacrificed and gastric tissue was processed for urease activity, H. pylori culture, histology, or immunohistochemistry, as described below.

Preparation of H. pylori whole-cell Ly Ags

H. pylori was grown on selective blood agar plates for 48 h at 37°C under microaerobic conditions (10% CO2, 5% O2, and 85% N2) and was suspended in PBS. The cells were then washed three times in PBS by centrifugation at 6000 rpm for 10 min at 4°C before being disrupted by freeze-pressing with the X-press (47). Briefly, The bacterial suspensions were frozen at −35°C in the form of cylindrical rods, which fit into the pressure chamber and were extruded by a piston forced back and forth by means of a hydraulic pump. After centrifugation (6000 rpm) to remove cell fragments, the preparation was filtered through a 0.2-μm membrane filter (Schleicher & Schuell, Dassel, Germany), and then the protein content was determined by the Bio-Rad (Hercules, CA) protein assay and aliquots were frozen at −70°C until they were used.

Gastric tissue analyses

The stomach was dissected along the greater curvature and divided into four strips for urease activity, H. pylori culture, histopathology, or immunohistochemical analyses.

For histopathology, longitudinal segments including the antrum and corpus plus a piece of attached intestine were fixed in 4% neutral buffered formaldehyde, embedded in paraffin, and sectioned at 3 μm by Histolab Products (Göteborg, Sweden). For immunohistochemistry, gastric sections were placed into Histocon (Histolab Products) at 4°C. The tissues were then placed in plastic forms (Cryomold; Miles, Elkhart, IN) filled with OCT compound (Miles) and were subsequently snap-frozen in isopentane in liquid nitrogen (N2) for ~60 s. Frozen cross-sections (5 μm) were prepared on microslides using a cryostat-1720 (Leitz, Wetzlar, Germany) and were frozen at −70°C.

Assessment of bacterial colonization

The presence of H. pylori in gastric tissue was assessed by urease activity measured spectrophotometrically using a colorimetric urease test (7). Antral segments (one quarter of the entire antrum), including corpus from each mouse, were placed in 0.5 ml of urea broth containing phenol red and were cultured as a whole piece of tissue for 8 h at room temperature. Thereafter, samples were vortexed and spun in a microcentrifuge for 1 min at 14,000 rpm, and 200 μl of the supernatant was used to determine the absorbance at 550 nm. For quantitative H. pylori culture of gastric tissue (7), longitudinal segments of gastric tissue were homogenized in 0.5 ml of bruccella broth supplemented with 5% FCS, and replicate serial 10-fold dilutions were plated on Helicobacter-selective blood agar plates. The plates were incubated at 37°C under microaerobic conditions (10% CO2, 5% O2, and 85% N2), and the quantitation of the CFU was performed 7 days later. In the present study, protection against H. pylori infection was defined as a significant reduction in the colonizing bacteria in the stomach.

Histopathology

For evaluation of gastritis, H&E-stained sections were scored based on the degree of infiltrating lymphocytes, plasma cells, and neutrophils (48). Grades were defined as follows: 0, none; 1, a few leukocytes scattered in the deep mucosa; 2, moderate numbers of leukocytes in the deep to mid mucosa and occasional neutrophils in gastric glands (microabscesses); 3, dense infiltrates in the deep to mid mucosa, a few microabscesses, and one or two lymphoid aggregates; and 4, dense, diffuse infiltrates throughout the lamina propria and into the submucosa, with prominent lymphoid aggregates, several microabscesses.

Immunohistochemistry of gastric tissue

Frozen sections from gastric tissue were fixed in 50% acetone for 30 s and then in 100% acetone for 5 min at 4°C. After washing in PBS, sections were blocked with avidin-biotin blocking reagents (Vector Laboratories, Burlingame, CA) and endogenous peroxidase activity was blocked with 0.3% H2O2 in PBS. Sections were then incubated with 5% horse serum in 0.1% BSA (Sigma-Aldrich) for 15 min in a humid chamber. Sections
were incubated with biotinylated rat mAbs against mouse CD4 or CD8 (BD PharMingen, San Diego, CA). For detection of CD3 cells, sections were incubated with rat mAb against mouse CD3 (BD PharMingen) followed by incubation with biotinylated rabbit anti-rat IgG (H + L; Vector Laboratories). Sections were then incubated with HRP conjugated to an avidin-biotinylated goat anti-rabbit Ig (ABC-Elite Kit; Vector Laboratories, Denemark). Cell-bound peroxidase was detected with 3-amino-9-ethylcarbazole (Vector Laboratories) and H2O2. Sections were counterstained with hematoxylin. Control sections were incubated without specific mAb. Stained sections were scored from 0 to 4 based on the extent of infiltrating T cells (30). Scores were defined as follows: 0, none; 1, a few T cells scattered in the mucosa; 2, moderate number of T cells in the gastric mucosa; 3, dense infiltrates in the deep to mid mucosa; and 4, dense, diffuse infiltrates throughout the lamina propria and into the submucosa.

Serum and gastric samples

Blood was obtained from the axillary plexus of the mouse at sacrifice. Gastric secretions were collected with absorbent wicks positioned longitudinally in the gastric lumen (49), after extensive rinsing with PBS containing 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Calbiochem, La Jolla, CA), 1 μg of aprotonin per ml, 10 mM leupeptin (Sigma-Aldrich), and 3.25 μM Bestatin (Boehringer Mannheim, Indianapolis, IN) protease inhibitor. For extraction of gastric secretions from the wick, 0.5 ml of protease inhibitor containing 5% nonfat dry milk was added to each sample tube containing two wicks, vortexed extensively, and then frozen at −70°C until it was analyzed.

Serum and gastric Ab determinations by ELISA

Because conflicting observations have been made as to the potential leakiness for Ab production in μMT mice, especially IgA, we undertook analyses of total and H. pylori-specific Ab levels in sera and gastric secretions from both μMT and WT mice (50, 51). Flat-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) were incubated with 5 μg/ml goat anti-mouse IgG or IgA (Southern Biotechnology Associates, Birmingham, AL) or with 10 μg/ml H. pylori Ly in PBS and were incubated at 4°C overnight. After washing and blocking with PBS containing 0.1% BSA, the wells were incubated with serial dilutions of sera or gastric secretions. The wells coated with Ly were then incubated with HRP-conjugated rabbit anti-mouse total Ig (DAKO; 1:3000) followed by incubation with o-phenylenediamine (Sigma-Aldrich) and H2O2 substrate in citrate buffer (pH 4.5), and the OD405 was determined using a Titertek Multiscan MS spectrophotometer (Labsystems, Stockholm, Sweden). For determination of isotype-specific Abs, the wells were incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG, IgG1, IgG2a, or IgA (Southern Biotechnology Associates; 1:500), followed by the phosphatase substrate p-nitrophenyl phosphate (Sigma-Aldrich) in ethanoldiamine buffer (pH 9.8), and the enzymatic reactions were read at 405 nm. Ab titters were defined on the linear portion of the curve as the interpolated dilution of a sample giving rise to an absorbance of 0.4 U above background. Whereas naive and immunized (Ly + CT) challenged WT mice had 2.4 ± 0.6 and 8.4 ± 1.0 mg/ml total IgG and 2.1 ± 0.6 and 7.9 ± 1.7 mg/ml IgA in sera, respectively, and 10.3 ± 4.9 μg/ml IgG and 154 ± 121.8 μg/ml IgA in gastric secretions (Ly + CT), no detectable IgG or IgA was found in the samples from μMT mice. The sensitivities of the total IgG and IgA ELISAs were 45 ng/ml and 170 ng/ml, respectively.

In vitro stimulation of spleen cells

Spleen cells were obtained by mechanical dissociation and filtering through a nylon mesh. RBCs were subjected to lysis by osmotic shock using hypotonic Tris-ammonium chloride. After washing in HBSS (Life Technologies, Paisley, UK), the cells were resuspended in Iscove’s medium (Biochrom) supplemented with 10% heat-inactivated FCS, 50 μM 2-ME (Sigma-Aldrich), 1 mM L-glutamine (Biochrom), and 50 μg/ml Gentamicin. Spleen cells (105 cells/well) were then cultured (8% CO2, 37°C) in round-bottom 96-well microtiter plates (Nunc) in the presence or absence of varying concentrations of H. pylori Ly Ags or anti-CD3 by adding 10% supernatant from the 145-2C12 cell line (52). Cell-free supernatants were collected 96 h after incubation and stored at −70°C until they were assayed for cytokine concentration.

Cytokine assays

The concentrations of IFN-γ, IL-4, IL-5, IL-10, or IL-13 in the supernatants were assessed by ELISA. Briefly, 96-well round-bottom microtiter plates (Dynatech Laboratories, Chantilly, VA) were incubated with 2.5 μg/ml rat anti-mouse IFN-γ (BD PharMingen), 1–5 μg/ml anti-mouse IL-4 (Endogen, Woburn, MA), IL-5, or IL-10 (BD PharMingen). The samples supernatants or recombinant mouse IFN-γ, IL-4, IL-5, or IL-10 (R&D Systems, Abingdon, U.K.) standards were then added to the appropriate wells. Bound IFN-γ, IL-4, IL-5, or IL-10 was detected by sequential incubations with a polyclonal rabbit anti-IFN-γ antisera or biotinylated mAb to mouse IL-4 (Endogen), IL-5, or IL-10 (BD PharMingen), followed by AP-conjugated goat anti-rabbit Ig (Southern Biotechnology Associates) for IFN-γ or HRP-conjugated anti-biotin (Vector Laboratories) for the IL-4, IL-5, and IL-10 assays. Finally, the AP substrate p-nitrophenyl phosphate in ethanoldiamine buffer (pH 9.8) or the HRP substrate H2O2 with ABTS (Sigma-Aldrich) in 0.1 M citrate buffer (pH 4.5) was added to each well, and the extent of the reaction was read at 405 nm using a Titertek Multiscan spectrophotometer. The cytokine concentrations in stimulated culture supernatants were estimated from the standard curves generated with each cytokine. IL-13 was determined using a mouse-specific IL-13 ELISA kit according to the manufacturer’s instructions (R&D Systems). The sensitivity of detection for the respective cytokines was as follows: 0.5 ng/ml for IFN-γ, 5 pg/ml for IL-4, 0.05 ng/ml for IL-5, 0.2 ng/ml for IL-10, and 1.5 pg/ml for IL-13.

Statistical analysis

Wilcoxon rank sum test was used for independent samples for analysis of significance.

Results

Primary H. pylori infection in μMT mice

Because it has been reported that specific Abs induced by H. pylori infection may cross-react with gastric mucosa and activate complement, we had reason to test whether the ability to produce Abs contributed to the development of inflammation and persistence of infection (12). To this end, groups of μMT or WT mice were infected intragastrically with 3 × 109 CFU of bacteria on two consecutive days, and the level of colonization and immunopathology was compared at intervals after inoculation. We found that at 2 wk after inoculation, the degree of colonization in μMT and WT mice was comparable, as assessed by urease activity (data not shown) and by quantitative culture (Fig. 1). However, at 8 wk after infection, μMT mice demonstrated a significantly (p < 0.001) reduced bacterial load compared with WT mice. In fact, 8-wk-infected μMT mice exhibited at least a 100-fold reduction in colonizing bacteria with a mean log10 value of 2.6 ± 2.8 vs 5.1 ± 2.2 in WT mice (data pooled from three experiments). Even at a later time, 16 wk, bacterial colonization was significantly lower in the μMT mice (Fig. 1).

To confirm the μMT status, we determined anti-H. pylori Ab production in serum and gastric secretion at 2 and 8 wk after H. pylori infection. No Abs were detected in the μMT mice, whereas WT mice demonstrated high levels of anti-H. pylori Abs (total Ig) in sera at 2 wk, which were then slightly increased by 8 wk of infection; log10 titers were 2.7 ± 0.4 and 3.0 ± 0.5, respectively. Undetectable or poor responses with anti-H. pylori Abs were seen in the gastric secretions of WT mice (data not shown). Because μMT mice have been reported to be leaky for non-IgM Ab production, especially IgA, we analyzed serum and secretions for total IgG or IgA, but found no detectable levels of IgG or IgA (see Materials and Methods) (50, 51). Taken together, μMT mice exhibited significantly enhanced clearance of H. pylori infection by 8 wk, suggesting that H. pylori-specific Abs augmented, rather than prevented, bacterial colonization.

Gastric inflammation and immunopathology in H. pylori-infected μMT mice

To assess the development of gastric inflammation in the Ab-deficient μMT mice, H&E-stained gastric sections were prepared and scored as described (48). As shown in Fig. 2A, the histopathology scoring indicated no inflammation at 2 wk in either the μMT or WT mice in response to a primary infection with H. pylori organisms. At 8 wk after inoculation, the WT mice exhibited only a mild
gastric inflammation, whereas the μMT mice, in contrast, were severely affected and demonstrated a substantial influx of inflammatory cells. Interestingly, eosinophilic leukocytes dominated and gave immunopathology scores significantly different from those in WT mice. The mean pathology score of three experiments performed at 8 wk after inoculation in the μMT mice was 2.9 ± 1.1 vs 1.1 ± 0.6 in the WT mice (p < 0.0001). At later time, 16 wk after inoculation, a reduction of the pathology score to 2.0 ± 1.3 was seen in the μMT mice (Fig. 2A). The massive infiltration of eosinophilic leukocytes in the μMT mice was mostly confined to the fundic mucosa close to the border of the upper part of the stomach with squamous epithelium. In fact, the inflammatory cells were mostly located in the deep mucosa above the muscularis mucosa and in the submucosa (Fig. 3). These findings indicate that a more severe gastric inflammation with eosinophilia develops in the absence of B cells and Abs. A correlation between the bacterial colonization and pathology score gave evidence of an inverse relationship between these parameters (Fig. 2B). Thus, the μMT mice had less colonizing bacteria but a more severe gastritis compared with WT mice, indicating that the lack of Abs aggravated gastritis but reduced the ability of H. pylori bacteria to colonize the mucosa.

Because T cells are known to play an important role in regulating inflammation, it was important to determine the distribution of T cells infiltrating the gastric mucosa. The densities of CD4+ and CD8+ T cells in the gastric mucosa were evaluated by immunohistochemistry. Both the μMT and WT mice had few T cells in the gastric mucosa at 2 wk after infection (Fig. 4). However, a significant infiltration of T cells, mostly CD4+ T cells, was observed in the gastric mucosa of μMT mice at 8 wk after inoculation (p < 0.001) (Fig. 4). At a later time (16 wk), we observed a continued increase in CD4+ T cells to a mean score of 2.3 ± 1.1 in the μMT mice. In contrast, few T cells were seen in the WT mice. The CD4+ T cells were localized in the lamina propria between gastric pits and in the deep mucosa and submucosa (data not shown).

Thus, at 8 wk and later the lack of Abs was associated with a lower level of bacterial colonization, more severe inflammation, and a pronounced infiltration of eosinophilic leukocytes and CD4+ T cells in the gastric mucosa of H. pylori-infected μMT mice as compared with the findings in WT mice.

Protection against H. pylori infection in μMT mice after oral immunization

Recent studies have demonstrated that mucosal immunization of μMT mice induces protection against a subsequent challenge with H. pylori organisms (8, 9). These studies, however, did not address the regulatory role of T cell subsets in protective immunity and in the immunopathology to a challenge infection with H. pylori or to what extent Abs influenced the postimmunization gastritis. In the present study, we found that μMT mice immunized orally with Ly and CT adjuvant exhibited significant protection against a challenge infection with H. pylori (p < 0.001) (Fig. 5). The protection was comparable with that induced in the immunized challenged WT mice. Both the μMT and WT mice exhibited a 100-fold reduction in bacterial load in the gastric tissue as compared with mice receiving CT or Ly alone (Fig. 5). Protection induced in the μMT and WT mice remained strong at 8 wk postchallenge (3.04 ± 0.58 vs 3.88 ± 1.3, not significant (NS), in the μMT and 3.26 ± 0.16 vs 5.94 ± 0.3, p < 0.001, in the WT; Ly + CT vs CT group) and even at 16 wk postchallenge (0.90 ± 0.62 vs 2.77 ± 1.21, NS, in the μMT and 1.53 ± 0.62 vs 4.61 ± 0.72, p < 0.01, in the WT; Ly + CT vs CT) (data not shown).

![Figure 1](http://example.com/figure1.png)

**FIGURE 1.** H. pylori colonization in μMT (filled bars) and WT (open bars) mice at 2, 8, and 16 wk after infection with live bacteria. Groups of 10 μMT and WT mice were infected with 3 × 10⁸ CFU of H. pylori SS1 (Sydney strain) on two consecutive days. The presence of H. pylori in gastric tissue was assessed by quantitative culture as described in Materials and Methods. The bars represent mean ± SEM of pooled data from two to three experiments. *, p < 0.001; †, p < 0.05; by Wilcoxon rank sum test.

**Gastric inflammation and immunopathology in immunized challenged μMT mice**

Histological evaluation of gastritis was performed on H&E-stained sections of gastric mucosa from the immunized challenged μMT and WT mice. As shown in Figs. 6A and 7, immunized μMT mice had less colonizing bacteria but a more severe gastritis compared with WT mice, indicating that the lack of Abs aggravated gastritis but reduced the ability of H. pylori bacteria to colonize the mucosa.

![Figure 2](http://example.com/figure2.png)

**FIGURE 2.** A. Gastric inflammation in μMT (○) and WT (○) mice after infection with H. pylori. The extent of histological gastritis was assessed 2, 8, and 16 wk after infection by evaluating H&E-stained sections from the gastric tissue as described in Materials and Methods. Data points are from individual mice. The bar represents the mean of each group. Data are pooled from two to three experiments. The mean ± SD scores in uninfected (PBS-treated) μMT and WT mice were 0.6 ± 0.8 and 0.1 ± 0.2, respectively. *, p < 0.0001, by Wilcoxon rank sum test. NS, Not significant. B. Mean gastritis score (as shown in A) vs mean CFU (as shown in Fig. 1) in the μMT and WT mice 8 wk after infection with H. pylori.
and WT mice demonstrated an eosinophil-rich inflammation at 2 wk after challenge, which was significantly higher than that in the control groups receiving CT or Ly alone. The postimmunization gastritis in the protected μMT and WT mice persisted at 8 wk postchallenge (2.55 ± 0.23 vs 2.42 ± 0.07, NS, in the μMT and 3.06 ± 0.18 vs 1.25 ± 0.35, p < 0.01, in the WT; Ly + CT vs CT) and even at 18 wk postchallenge (2.94 ± 0.24 vs 2.00 ± 0.35, NS, in the μMT and 2.33 ± 0.29 vs 2.11 ± 0.35, NS, in the WT; Ly + CT vs CT). A strong infiltration of CD4⁺ T cells in the gastric mucosa of immunized μMT and WT mice was observed at 2 wk postchallenge, whereas only a few T cells were seen in unprotected μMT and WT mice immunized with CT or Ly alone (Fig. 6B). CD4⁺ T cell infiltration in the protected μMT and WT mice remained strong even at 18 wk postchallenge (1.60 ± 0.39 vs 1.00 ± 0.42, NS, in the μMT and 2.60 ± 0.20 vs 0.90 ± 0.28, p < 0.001, in the WT; Ly + CT vs CT); CD4⁺ T cells were localized in the lamina propria between gastric pits and in the deep mucosa and submucosa (Fig. 7).

As seen in previous studies, oral immunization of WT mice with _H. pylori_ Ly plus CT resulted in the development of high levels of anti- _H. pylori_ IgG1 and IgG2a Abs in sera and gastric secretions at 2 wk postchallenge (data not shown). The mean IgG1 and IgG2a Ab titers (log₁₀) were 4.2 ± 0.5 and 3.6 ± 0.6 in sera as well as 1.1 ± 0.4 and 0.6 ± 0.6 in gastric secretions, respectively. Anti- _H. pylori_ IgG1 and IgG2a titers remained strong in the protected WT mice at 8 and 18 wk postchallenge (data not shown). At 2 wk postchallenge, anti- _H. pylori_ IgA Abs were not detected in gastric secretions and only poor serum responses were detected (data not shown). At 8 and 18 wk postchallenge, however, high levels of anti- _H. pylori_ IgA Abs were detected in sera and gastric secretions of protected WT mice. The mean IgA Ab titers in sera at 8 and 18 wk were 2.9 ± 0.6 and 2.4 ± 0.3, and in gastric secretions they were 1.7 ± 0.3 and 2.1 ± 0.3, respectively. As expected, no Abs were detected in the μMT mice. Thus, we observed no difference between μMT and WT mice with regard to the level of protection or the degree or quality of inflammation, suggesting that resistance to bacterial colonization correlates positively with gastric inflammation but does not correlate with Ab production. Therefore, specific Abs are not involved in the development or maintenance over time of postimmunization gastritis.

**Th1 and Th2 cytokine responses in immunized and protected μMT mice**

Next we analyzed the cytokine production of T cells in well-protected mice. Splenocytes were isolated and cultured in the presence or absence of recall _H. pylori_ Ly Ags. T cells from protected, immunized challenged, μMT, and WT mice produced elevated levels of IFN-γ at 2 wk that remained high even at 8 wk postchallenge (Table I). Also, IL-13 was detected at 2 wk and increased at 8 wk postchallenge. In contrast, IL-4 production was detected first after 8 wk in μMT mice and to a lesser degree in WT mice (Table I). IL-5 production was below the level of detection at all times and IL-10 was seen at low levels only at 8 wk postchallenge in WT mice. Thus, immune protection against _H. pylori_ infection was associated with a predominance of Th1 type response at 2 wk, but at 8 wk postchallenge, a mixed Th1 and Th2 response was found.
In the present study, we have demonstrated that Abs not only are dispensable for protection, but they appear to be detrimental to elimination of the bacteria in WT mice. In fact, resistance against infection in the absence of Abs seems a lot more effective than when Abs are produced. Contrary to WT mice, we observed that the μMT mice nearly cleared the infection by 12–16 wk postinoculation. At 8 wk, their gastric mucosa showed severe inflammation with recruitment of large numbers of eosinophilic leukocytes and CD4⁺ T cells. The gastritis subsided by 16 wk as the bacterial counts dropped, whereas, by contrast, WT mice exhibited extensive bacterial colonization and relatively mild inflammation at this time. Also, WT mice followed out over time (>16–32 wk) can significantly reduce their bacterial load as shown by Garhart et al. (32), but the kinetics are much slower compared with those observed in μMT mice. Thus, Abs prolong the time for elimination of bacteria (45).

Discussion
The role of Abs in the resistance against H. pylori infection has been debated for more than a decade (8, 14, 39–41, 53–55). In experimental models, evidence has been presented that both supports and rejects a role for Abs in protection against infection (8, 9, 39, 42, 44, 55). However, not until recently have we been able to adequately address the question of whether specific Abs have a protective role or not by comparing the bacterial colonization after challenge with live bacteria in mice that are deficient in all Ig (μMT mice) or in IgA only and in WT control mice (8–10, 43, 44).

Of the published papers using μMT mice, most have focused on protection against infection in well-immunized mice and all have found that Abs appear not to be essential for protection (8–10, 43, 44). Based on these and other studies, Abs have been considered a correlate rather than an effect of protective immunity. However, few of the studies have analyzed systemic and local Ab production in light of the gastric immunopathology that most often is associated with resistance against H. pylori infection (8, 9, 44).

In the present study, we have demonstrated that Abs not only are dispensable for protection, but they appear to be detrimental to elimination of the bacteria in WT mice. In fact, resistance against infection in the absence of Abs seems a lot more effective than when Abs are produced. Contrary to WT mice, we observed that the μMT mice nearly cleared the infection by 12–16 wk postinoculation. At 8 wk, their gastric mucosa showed severe inflammation with recruitment of large numbers of eosinophilic leukocytes and CD4⁺ T cells. The gastritis subsided by 16 wk as the bacterial counts dropped, whereas, by contrast, WT mice exhibited extensive bacterial colonization and relatively mild inflammation at this time. Also, WT mice followed out over time (>16–32 wk) can significantly reduce their bacterial load as shown by Garhart et al. (32), but the kinetics are much slower compared with those observed in μMT mice. Thus, Abs prolong the time for elimination of bacteria (45).
The reasons for this can be severalfold. First, Abs may dampen the development of inflammation in response to a primary infection with *H. pylori* bacteria (56). A neutralizing effect of Ab on the presence of proinflammatory factors released by the bacterium could contribute to dampening of the inflammation (57). Alternatively, specific Abs, especially secretory IgA that does not activate complement, may bind to the bacteria in the gastric lumen and thereby allow the bacteria to evade immune recognition (17, 58, 59). In humans it has been documented that a majority of local IgA production in *H. pylori*-induced gastritis is not polymeric and is of the IgA1 subclass, also largely lacking J-chain, which impairs the active transmembrane transport of IgA (60, 61). The IgA that is present in the gastric lumen in the gastritis thus would be unstable, susceptible to degradation by proteases, and masking rather than identifying the presence of the pathogen. It is of note, however, that *H. pylori* appears to lack the ability to produce an IgA1 protease, which is commonly made by several other species and which could degrade the protective IgA1, but not IgA2 (60). Moreover, a deposition of IgA and IgG Abs on the bacteria below a critical threshold may also avoid activation of an inflammatory response and in this capacity may impair the elimination of the bacteria (62). Binding of Ab to leukocyte FcγRs with an inhibitory function and promoting the release of anti-inflammatory cytokines, such as IL-10, may be other mechanisms for inhibition of local inflammation (63, 64). Secondly, Abs may facilitate bacterial adherence to the mucus layer covering the gastric epithelium, thereby promoting rather than preventing a hospitable environment for the colonizing bacteria. Such an effect, for example, has been reported with Abs against the capsular polysaccharide of *Pneumococci*, which exposed the ligand for phosphorylcholine facilitating bacterial adherence to epithelial cells (65). In agreement with this notion, we observed that bacterial colonization increased concomitant with increased production of specific Abs in the WT mice. Notably, chronically infected patients have developed high titers of specific IgA and IgG in the serum and gastric mucosa, but can remain

**FIGURE 7.** Gastric pathology and T cell infiltration 2 wk after challenge in the immunized μMT and WT mice. Photomicrographs of H&E-stained sections of gastric mucosa and submucosa from μMT (A and C) and WT (B and D) (bars = 20 μm) mice immunized with Ly and CT showing a massive infiltration of inflammatory cells, mostly eosinophils in the upper submucosa and also in the mucosa (arrows). T cell infiltration was determined by immunohistochemistry, and a significant accumulation of CD4+ T cells (arrows) was observed in the gastric mucosa of μMT mice immunized with Ly and CT (E), whereas only a few T cells were observed in the CT-treated control mice (F) (bar = 20 μm). A significant accumulation of CD4+ T cells was also observed in the immunized challenged WT mice (not shown).
infected for a lifetime (66). Thus, it appears that Abs may counteract resistance to infection with *H. pylori* bacteria.

The postimmunization gastritis phenomenon correlated well with immune protection in the present study. This is not unique and several groups have reported strong links between the level of protection against *H. pylori* infection and gastritis in other models, but this study is the first to demonstrate that Abs are not required for this phenomenon to occur (32). We found comparable gastritis in immunized WT and μMT mice upon challenge with live *H. pylori* bacteria. The implications of this observation are important and argue that T cells are responsible for the gastritis. The findings of Roth et al. (45) lend support to this idea in that they documented cross-reactive autoaggressive Abs (11, 12); rather, it is the result of a postimmunization phenomenon is not the result of production of inflammatory cells may play a role in resistance against migration larvae (70). Although IL-5, together with its impact on resistance against *H. pylori* infection.

The strong link between gastritis and protection may demonstrate a cause and effect relationship. However, we and others have made observations to indicate that this is not a simple relationship (30, 32). It has become clear that immune protection does not prevent colonization with bacteria upon challenge, but it reduces and limits the extent of colonization. In this regard, protection in immunized mice reflects the same pattern as that observed after a primary infection of μMT mice. The infiltration of CD4+ T cells in the gastric mucosa appears to be a common denominator between different studies reporting on protection against *H. pylori*

### Table I. Cytokine production by spleen cells from the immunized challenged μMT and WT mice<sup>a</sup>

<table>
<thead>
<tr>
<th>Mice</th>
<th>Cytokine</th>
<th>Lysate + CT</th>
<th>CT only</th>
<th>Lysate + CT</th>
<th>CT only</th>
</tr>
</thead>
<tbody>
<tr>
<td>μMT</td>
<td>IFN-γ (ng/ml)</td>
<td>15.5 ± 2.5</td>
<td>0</td>
<td>13.7 ± 2.3</td>
<td>2.2 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>IL-4 (pg/ml)</td>
<td>0</td>
<td>0</td>
<td>112 ± 16.2</td>
<td>32.5 ± 22.7</td>
</tr>
<tr>
<td></td>
<td>IL-5 (ng/ml)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IL-10 (ng/ml)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IL-13 (pg/ml)</td>
<td>50.3 ± 18</td>
<td>0</td>
<td>607.1 ± 242.7</td>
<td>105.7 ± 56.2</td>
</tr>
<tr>
<td>WT</td>
<td>IFN-γ (ng/ml)</td>
<td>13.7 ± 0.7</td>
<td>0</td>
<td>6.1 ± 2.5</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>IL-4 (pg/ml)</td>
<td>0</td>
<td>0</td>
<td>5.5 ± 2.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IL-5 (ng/ml)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>IL-10 (ng/ml)</td>
<td>0</td>
<td>0</td>
<td>1.0 ± 0.4</td>
<td>0.2 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>IL-13 (pg/ml)</td>
<td>69 ± 43.9</td>
<td>0</td>
<td>279.8 ± 54.5</td>
<td>32.6 ± 25.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Groups of μMT and WT mice were immunized orally with *H. pylori* lysate together with CT or CT alone and then challenged with *H. pylori* bacteria. At 2 and after 8 wk postchallenge, spleen cells were isolated and cultured in the presence or absence of 2 μg/ml of *H. pylori* lysate Ags. Cell-free supernatants were collected 96 h later and analyzed in triplicate for the concentration of cytokines produced by T cells incubated with lysate minus the concentration of cytokine produced by T cells incubated in medium. Values are expressed as mean ± SEM of three pairs of mice per group and represent two to three experiments.
in particular Th1 cells, rather than specific Ab production. Such a vaccine could best be developed if based on peptide sequences that usually are poorly recognized by Ab (29). The protective peptide sequences could be identified based on TCR recognition using, for example, gene libraries of H. pylori-specific T cell clones and molecular modeling (78). Work along these lines is largely missing today. We propose that vaccine development against H. pylori may be a good example of where selective priming of CD4 T cell immunity should be attempted, rather than global approaches using whole organism, protein, or DNA-based vaccine strategies.

References