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Cellular Immune Responses Are Essential for the Development of Helicobacter felis-Associated Gastric Pathology

Kevin A. Roth,* Sharookh B. Kapadia,* Steven M. Martin,* and Robin G. Lorenz*‡§

The bacteria Helicobacter pylori is a major human pathogen that infects over half of the world’s population. Infection initiates a series of changes in the gastric mucosa, beginning with atrophic gastritis and leading in some patients to peptic ulcer disease, mucosa-associated lymphomas, and gastric adenocarcinoma. Although this cascade of events clearly occurs, little is known about the role of the host immune response in disease progression. We have utilized the C57BL/6 Helicobacter felis mouse model to critically analyze the role of the adaptive immune response in the development of Helicobacter-associated gastric pathology. Infection of B and T cell-deficient RAG−/− mice or T cell-deficient TCRβ−/− mice with H. felis resulted in high levels of colonization, but no detectable gastric pathology. Conversely, infection of B cell-deficient μMT mice resulted in severe gastric alterations identical with those seen in immunocompetent C57BL/6-infected mice, including gastric mucosal hyperplasia and intestinal metaplasia. These results demonstrate that the host T cell response is a critical mediator of Helicobacter-associated gastric pathology, and that B cells and their secreted Abs are not the effectors of the immune-mediated gastric pathology seen after H. felis infection. These results indicate that in addition to specific Helicobacter virulence factors, the host immune response is an important determinant of Helicobacter-associated disease. The Journal of Immunology, 1999, 163: 1490–1497.

It has been 14 years since Helicobacter pylori was conclusively shown to cause acute gastritis (1, 2). Patients infected with H. pylori develop a chronic gastric inflammatory infiltrate that primarily affects the antral region. The majority of infections result in no clinical symptoms; however, 10–20% progress to serious clinical disease. Multiple studies have demonstrated that H. pylori is associated with the development of duodenal and gastric ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma (3–5). The critical factors, either host, bacterial, and/or environmental, that influence the clinical manifestations of Helicobacter infection are still being elucidated. Some of the observed clinical variation in the pathologic response to H. pylori is due to phenotypic and genotypic diversity of the H. pylori bacteria itself, as strains of H. pylori with the cytotoxin-associated gene (cag)3 pathogenicity island have been associated with more severe peptic ulcer disease (6). However, this correlation does not account for all the manifestations of Helicobacter-induced disease, as MALT lymphomas are not associated with expression of the cag pathogenicity island, and in Far Eastern countries no association between disease outcomes and cytotoxin genes has been demonstrated (7, 8). These observations suggest that additional factors must interact with H. pylori to influence the course of gastric disease.

One potential host factor is the adaptive immune response, as the gastric inflammatory infiltrate associated with Helicobacter infection is predominantly IgG- and IgA-producing B cells and CD4 and CD8-positive T cells (9, 10). Initial studies in immunodeficient (C.B-17 SCID) mice indicated that the adaptive immune response was not required to maintain chronic gastric inflammation after Helicobacter infection (11). However, recent reports have now shown that several mouse strains, including the strain used above, do not develop gastric pathology after Helicobacter infection. Specifically, the inbred mouse strain C57BL/6 develops a severe chronic active gastritis and intestinal metaplasia, whereas BALB/c mouse strains exhibit only mild gastritis after infection with the H. pylori strain SS1 (Cag-A+) and the closely related Helicobacter felis (Cag-A-) (12–15). Due to the availability of this new C57BL/6 mouse model of Helicobacter-associated gastric disease, we reevaluated the hypothesis that the host adaptive immune response initiated by Helicobacter infection is responsible for subsequent gastric pathology.

We established gastric infection with H. felis in C57BL/6 wild-type and induced mutant mice. H. felis infection in C57BL/6 mice mimics human disease in many aspects, including a predominant CD4+ T cell response (9, 16) that is associated with an increased epithelial cell proliferative index and hyperplasia (12, 13, 17). However, H. felis does not express the cag pathogenicity island or the vacuolating toxin (VacA), and thus does not itself damage the gastric epithelium (18). Induced mutant mouse strains deficient in both B and T cells, or in B cells or T cells alone were infected with H. felis, and the subsequent gastric pathology was evaluated. In this report, we demonstrate that the T cell response is crucial for Helicobacter-induced gastric pathology, and that post-Helicobacter infection gastric destruction and hyperproliferation are not mediated by B cells or their secreted products.

Materials and Methods

Mice

C57BL/6-J/Rag−/−Igμ−/−, C57BL/6-μMT, and C57BL/6-Tcrβ−/− breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME). The strains were maintained by mating homozygous
siblings. C57BL/6/J control mice were purchased from The Jackson Laboratory. Mice were housed in a specific pathogen-free facility in microisolator cages under a strictly controlled light cycle and given a standard autoclaved chow diet ad libitum. All mice were maintained in accordance with the guidelines of the Animal Studies Committee of Washington University.

**Generation of H. felis-induced gastritis**

*Helicobacter* (ATCC 49179; American Type Culture Collection, Manassas, VA) was grown on moist ATCC 260 medium (trypticase soy agar (BBL 11043; Fisher Scientific, Pittsburgh, PA)) supplemented with defibrinated calf blood (5% v/v; Colorado Serum Company, Denver, CO), trimethoprim (5 mg/L; Sigma, St. Louis, MO), vancomycin (6 mg/L; Sigma), and Fun-gizone (1% v/v; Life Technologies, Gaithersburg, MD) under microaerophilic conditions at 37°C for 2 days. Confluent plates of *H. felis* were harvested, and the number of bacteria were determined by adsoption at OD _450_ with one OD unit corresponding to 10^9_ bacteria. Bacteria were stored in a mixture of brain heart infusion broth (BHI; Difco 0037; Fisher) and glycerol (31 ml glycerol/100 ml broth) at −70°C. Per os (p.o.) infection of *H. felis* was accomplished using a 200-μl pipette tip. Mice were inoculated twice over a 3-day period with 0.05 ml of bacterial suspension. Mock infected animals were inoculated with the BHI/glycerol suspension medium.

**Assessment of H. felis colonization and histopathology**

Mice were sacrificed and their stomachs rapidly removed. Ninety minutes before sacrifice, animals received an i.p. injection of 5-bromo-2′-deoxyuridine (BrduRad; Sigma) (dose, 120 mg/kg) to label cells in S phase (19). Tissue samples were fixed in methacarn solution (60% methanol, 30% chloroform, 10% glacial acetic acid) and embedded in paraffin, and 5-μm-thick sections were prepared. Colonization was established by evaluation of immunohistochemical staining of the *H. felis* organisms in histologic sections of the entire greater and lesser curvatures of the stomach. A semi-quantitative determination (*H. felis* (HF) colonization score) of the organism load was made based on the number of bacteria observed per gastric glandular unit (0 = no bacteria, 1 = 1–2 bacteria, 2 = 3–10 bacteria, 3 = 11–20 bacteria, 4 = >20 bacteria). Sections were evaluated by two pathologists blinded to the experimental status of the mice.

Gastrointestinal inflammation and destruction were assessed by microscopic examination of hematoxylin/eosin-stained gastric tissue. The stomachs were transected along the greater and lesser curvature to obtain two halves, with each half subsequently embedded in paraffin. Gastric histopathology was evaluated by two pathologists blinded to the strain and experimental status of the mice. The scoring system used was modified from Ernark et al. (20) and is based on a grade of 0–3 in each of three categories: longitudinal extent of inflammation (0 = no inflammation, 1 = patchy, 2 = <50%, 3 = >50%); vertical extent of inflammation (0 = no inflammation, 1 = basal lamina propria only, 2 = transmural, 3 = both mucosa and submucosa involvement); and histopathological changes (0 = none, 1 = mild alterations in differentiated epithelial cells, 2 = moderate alterations in differentiated epithelial cells, 3 = severe alterations in differentiated epithelial cells). The total histological score (0–9) was determined by summation of each subscore. All zones of the stomach were examined: squamous zone, zymogenic zone (parietal cells, zymogenic cells, surface and neck mucous cells), mucoparietal zone (parietal and zymogenic cell density, and inflammation (23). Specifically, a panel of lectins and Abs was used as previously described to define parietal and zymogenic cell density, and inflammation (23). Briefly, 5-μm-thick sections were deparaffinized, nonspecific staining blocked using PBS containing 1% BSA, 0.2% powdered skim milk, and 0.3% Triton X-100 (PBS-BB), and then incubated with the appropriate primary Ab diluted in PBS-BB overnight at 4°C. After extensive PBS washes, cyanine (Cy)-3 or fluorescein (FITC)-conjugated secondary Abs and/or fluorescent-conjugated lectins were added, incubated on the sections for 1 h at room temperature, washed and covered slipped with PBS/glycerol (1:1), and examined using a Zeiss (Oberkochen, Germany) Axioskop fluorescent microscope. The methods used for single- and double-label immunohistochemistry have been described previously (26). Abs and lectins used were: goat anti-BrdUrd (final dilution, 1:1000; kindly supplied by Dr. Steven Cohn, University of Virginia, Charlottesville, VA) (21, 22); rabbit anti-rat intrinsic factor (1:1000) (27); rabbit anti-rat pepsinogen (1:500, kindly supplied by Michael Samloff, University of California, Los Angeles, CA) rabbit anti-H^+^-/K^+^-ATPase β-subunit (1:250, kindly supplied by Michael Caplan, Yale University, New Haven, CT); rabbit anti- H. pylori (1:1000, Dako, Carpinteria, CA); *Griffonia simplicifolia* II lectin (specific for mucous neck cells; final concentration, 5 μg/ml, EY Laboratories, San Mateo, CA) (24); *Dolichos biflorus* agglutinin (specific for parietal cells; final concentration. 20 μg/ml; Sigma) (24); cholera toxin B-subunit (specific for surface mucus (pit) cells; final concentration, 5 μg/ml; List Biological Laboratories, Campbell, CA) (24).

**Statistical analysis**

Data analysis using a standard Student’s t test or Mann-Whitney test was performed using GraphPad Prism (San Diego, CA).

**Results**

Adaptive immunity is critical for Helicobacter-associated gastric pathology

To assess the contribution of the adaptive immune response to the gastric epithelial pathology seen after *Helicobacter* infection, C57BL/6 and C57BL/6-Rag1<sup>−/−</sup>Mom (RAG-1<sup>−/−</sup>) mice were infected p.o. with *H. felis*. RAG-1<sup>−/−</sup> mice have no mature B or T lymphocytes and consequently do not mount any Ag-specific immune responses (28). Infected mice and control mock-infected mice were sacrificed 4, 6, 8, 14, and 22 wk after inoculation (14 total C57BL/6 and 15 RAG-1<sup>−/−</sup>). After 4 wk of infection, the RAG-1<sup>−/−</sup> mice had similar levels of *Helicobacter* organisms in their gastric glands as C57BL/6, with colonization initially occurring in the pure mucous zone (corresponding to the antral region of the human stomach) (Fig. 1). However, at all time points examined, they displayed minimal immune infiltrates, no alterations in their gastric morphology (Fig. 2, A–C) and no proliferative abnormalities (data not shown). This was in contrast to the C57BL/6 immunocompetent mice, which as early as 4 wk after infection displayed gastric infiltrates consisting primarily of mononuclear cells.
and scattered neutrophils. This infiltrate was accompanied by dramatic gastric epithelial changes, including a marked reduction in the number of parietal and zymogenic cells in the gastric epithelium by 14 wk after inoculation with *H. felis* (Fig. 2, C, D, and F). These severe alterations in differentiated gastric epithelial cells are reflected by the high histopathological changes subscore as seen in Table I. The differentiated gastric epithelial cells appear to be replaced by two cell types. One is a rapidly proliferating cell that does not display any characteristics of gastric epithelial cell differentiation (see Fig. 5B). The second is a mucous-producing goblet-like cell that results in a histologic picture characteristic of intestinal metaplasia (see Fig. 3, E and F). The absence of gastric pathology in *H. felis* infected RAG-1−/− mice was consistently seen at all time points examined, even though all RAG-1−/− animals maintained high levels of *H. felis* colonization (Fig. 1). These findings indicate that in the C57BL/6 inbred mouse strain, the host adaptive immune response generated after infection with *Helicobacter* was causally responsible for the subsequent gastric pathology.

**T cells, not B cells, are the crucial mediators of *H. felis*-associated gastric pathology**

Recent reports have demonstrated that infection with *H. pylori* can lead to the production of anti-*H. pylori* Abs that cross-react with human gastric mucosal Ags (29–34). These autoantibodies have the potential to play a crucial role in the pathogenesis of gastric diseases, as Negrini et al. (29) demonstrated that mice bearing mature B cells. The pathology seen after *H. felis* infection of B cell-deficient mice was identical with that seen in wild-type C57BL/6 (Fig. 2, E, H, and I). As early as 4 wk after inoculation with *H. felis*, a severe inflammatory infiltrate, consisting of both monocytes and neutrophils, was seen. This infiltrate was accompanied by dramatic epithelial changes, including a marked reduction in zymogenic and parietal cells (Fig. 3, A and D, and data not shown). The differentiated gastric epithelial cells were replaced by a hyperplastic epithelium, which included large areas of intestinal metaplasia containing mucin-producing goblet cells (Fig. 3E). These epithelial cell alterations were seen in both the C57BL/6 and µMT mouse strains and were maintained throughout the length of infection (20 wk, a total of 32 µMT and 29 C57BL/6 mice were examined). Both mouse strains appeared to drastically reduce their levels of *H. felis* colonization by 12 wk after inoculation (Fig. 1), demonstrating that B cells and/or Abs are not essential for this reduction in bacterial colonization levels.

*H. felis* infection of TCR (TCRβ) double knockout mice (*n* = 28; C57BL6/J-Tcrβ∆/∆−/− mice) did not result in any significant histological changes when compared with mock-infected control TCRβ−/− mice. Mice examined 4–12 wk after *H. felis* inoculation had high levels of bacterial colonization (Fig. 1), but demonstrated minimal gastritis and no significant gastric epithelial pathology (Fig. 4 and Table I). It should be noted that mock-infected control TCRβ−/− mice have a low, but consistent level of gastric inflammation and histological changes. As these TCR mutant mice have been previously described to develop spontaneous inflammatory bowel disease, this low level gastric inflammation is not surprising (37). Although *H. felis* infection significantly increases the total histology score in the TCRβ−/− mice; this elevated score is due to an increase in the level of inflammatory infiltrate, as the histopathological changes subscore is not significantly different (Table I). These data indicate that T cells and/or their secreted products are the crucial mediators of the gastric pathology seen after *Helicobacter* infection.

**Gastric epithelial cell proliferation was increased in response to *H. felis* infection**

Intestinal-type gastric cancer develops over decades from superficial to chronic gastritis, followed by the development of atrophy, intestinal metaplasia, dysplasia, and finally cancer (39). We have now demonstrated that the initial stages of this pathway—gastritis and atrophy (defined as the loss of parietal and chief cell populations)—are a result of the T cell response to *Helicobacter* infection. As gastritis and atrophy are associated clinically with an increase in mucosal cell proliferation (40, 41), we wanted to directly assess the levels of gastric epithelial cell proliferation in our mouse model of *Helicobacter* gastritis. The mice were injected i.p. with C57BL/6 mice with an isolated deficiency in the B cell compartment caused by a targeted disruption in the heavy chain of IgM (C57BL/6-Igh-6mIgM−, µMT) (35). This disruption results in B cell maturation arrest at the pre-B cell stage and the absence of mature B cells. The pathology seen after *H. felis* infection of B cell-deficient mice was identical with that seen in wild-type C57BL/6 (Fig. 2, E, H, and I).
BrdUrd (120 mg/kg) 90 min before sacrifice. This thymidine analogue is incorporated into the DNA of dividing cells. Cells in S phase during the injection period can subsequently be immunohistochromically detected in tissue sections using an Ab specific for BrdUrd (19, 21). In normal murine stomach, BrdUrd labeling was limited to a narrow band of positive cells in the previously well described isthmus progenitor cell zone (42). In contrast, *H. felis*–infected C57BL/6 stomachs demonstrated a dramatic increase in the number of BrdUrd-positive cells and in the width of the proliferative zone (Fig. 5, A and B, and Table II). The marked proliferative abnormalities were seen as early as 6 wk after infection in C57BL/6 mice and continued throughout the length of our experiment (20 wk). Note, that after 12 wk, there were very few detectable *Helicobacter* organisms in the stomach (Fig. 1); thus, direct effects of *H. felis* itself on gastric proliferation is unlikely.

Epithelial cell hyperplasia was not dependent on the presence of B cells, as a marked expansion of the number of gastric epithelial cells in S phase as well as the size of the proliferative zone was observed in infected B cell-deficient μMT mice (Fig. 5, C and D, and Table II). In contrast, there was no proliferative abnormality seen at any time after infection in the RAG-1−/− mouse strain or in uninfected control mice (data not shown).
Discussion

In this study, we used the induced mutant mouse strains RAG-1 \(-/-\), TCR\(\beta\) \(-/-\), and \(\mu\)MT to evaluate the contribution of the adaptive immune response to the gastric epithelial hyperproliferation, altered glandular differentiation, and intestinal metaplasia seen after *Helicobacter* infection. C57BL/6 mice demonstrated severe chronic active gastritis with increased epithelial cell proliferation and loss of specialized parietal and zymogenic cells after *Helicobacter* infection. Strikingly, C57BL/6 mice with either no mature B or T cells (RAG-1 \(-/-\)) or specifically no mature T cells (TCR\(\beta\) \(-/-\)) do not develop gastric epithelial pathology after *H. felis* infection. Although immune responses have been postulated to be involved in the progression from *Helicobacter* infection to gastric atrophy, metaplasia, and cancer, this is the first demonstration of the crucial role of the T cell response in this progression. The hyperplastic epithelium and altered glandular differentiation seen in the C57BL/6 mouse are all features of preneoplastic lesions seen in the recently described Mongolian Gerbil model of *Helicobacter*-induced gastric cancer (43).

These results confirm and extend our previous report that targeted microbial attachment in a novel transgenic mouse model of *H. pylori* infection results in increased gastric infiltration and gastric pathology (44). These data also serve as a potential explanation for the observation of an unexpectedly low prevalence of *H. pylori*-associated gastric histopathology in immunocompromised patients with AIDS (45). However, our findings are in contrast with...
a previously reported H. felis SCID model, which concluded that the host adaptive immune response played a limited role in control of Helicobacter colonization (11). In the SCID study, immunodeficient (SCID) mice and immunocompetent C.B-17 congenic mice (identical with the BALB/c strain except at the Ig heavy-chain locus (Igh) (46) were both infected with H. felis. No statistical difference between the two mouse strains in the number of H. felis organisms or the intensity of inflammation was seen; however, this study did not evaluate Helicobacter-associated gastric pathology. We and others have now shown that the BALB/c inbred mouse strain does not develop extensive gastric pathology after Helicobacter infection (histological score = 2.05 ± 0.43, 4–28 wk after infection, n = 10), whereas the C57BL/6 mouse strain develops hyperplasia and parietal and zymogenic cell destruction (see Fig. 2) (12, 13). Therefore, to stringently test our hypothesis that the host adaptive immune response initiated by Helicobacter infection is causally responsible for the subsequent gastric pathology, it was necessary to infect immunodeficient mice that were of the C57BL/6 background. The different interpretation of our results and the previously published data is almost certainly due to our ability to detect and quantitate gastric mucosal abnormalities in the C57BL/6 strain, as these abnormalities do not occur in the C.B-17/BALB/c inbred strain.

It has been clearly shown that human infection with H. pylori induces autoantibodies reactive with gastric parietal cell Ags, specifically to H/K-ATPase, which is also known to be a key autoantigen in autoimmune gastritis (34, 47). It is unknown whether these autoantibodies are directly pathogenic for the gastric epithelium or are in response to gastric epithelial cell destruction caused by Helicobacter virulence factors such as cytotoxins (48). The significance of autoantibodies to Helicobacter-induced gastric pathology has proven difficult to experimentally address in human subjects. To assess the contribution of host B cells and their products in Helicobacter-induced gastric pathology, we have infected the B cell-deficient mouse strain, μMT. Our results showed that the μMT and C57BL/6 mice exhibited identical gastric pathology after H. felis infection, with mucosal inflammation, gastric epithelial cell hyperproliferation, parietal and zymogenic cell destruction, and metaplastic changes. This demonstrates for the first time that B cells and their secretion of potentially autoreactive Abs are not critical to the development of gastric epithelial cell destruction and hyperplasia after H. felis infection.

The finding that the adaptive immune system, specifically T cells, are critical mediators of H. felis-associated gastric pathology does not allow for the discrimination between direct Ag-specific and indirect non Ag-specific effects of T cells. There are at least two major potential mechanisms of T cell-dependent gastric pathology. One mechanism would be based on the postulate that specific immune recognition events are critical for the resulting gastric epithelial pathology. This Ag-specific event, where a T cell with a TCR specific for gastric self-proteins could be stimulated to develop by molecular mimicry of a H. felis protein, or by exposure to a previously sequestered gastric Ag, could result in the development of a gastric autoimmune disease. A second potential mechanism is based on an indirect effect of T cells and the factors they secrete in response to a gastric infection. In this scenario, the T cell response to H. felis infection results in a cytokine/chemokine milieu that modifies the differentiation of gastric epithelial cells from the gastric epithelial stem cell. This altered milieu could be due either to the infiltration of H. felis-specific T cells or to the influx of a polyclonal T cell population into the gastric epithelium. This modified gastric epithelial cell differentiation program would result in an increase in proliferating undifferentiated epithelial cells and mucus-producing intestinal-like epithelial cells, and a decrease in zymogenic/parietal cells. As there is currently a very limited knowledge of the factors involved in gastric epithelial differentiation from the gastric stem cell, it is unclear whether lymphocyte-derived factors could have this effect on gastric epithelial cell differentiation.

There is now evidence in human H. pylori infection of an altered gastric epithelial cell turnover early in the course of the disease (49–51). Both increased epithelial cell apoptosis and proliferation have been described. The lack of epithelial cell hyperproliferation in the RAG-1−/−-infected mice indicates that in the absence of the adaptive immune response, factors are missing that crucially affect epithelial cell proliferation. These data suggest that future investigations of the infiltrating adaptive immune response will further our knowledge of the mechanisms by which Helicobacter infection results in hyperplastic changes in the gastric epithelium.

In summary, our studies have demonstrated that the host T cell response initiated by Helicobacter infection is causally responsible for subsequent gastric pathology. Future studies of the interplay between the host cellular immune response and Helicobacter infection should provide insights into the key immune mediators responsible for the Helicobacter-associated gastric pathology.

Acknowledgments

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References


Table II. Proliferative abnormalities and hyperplasia in mice infected ≥ 8 wk with Helicobacter felis

<table>
<thead>
<tr>
<th>Strain</th>
<th>BrdUrd Cells</th>
<th>Mucosal Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 mock</td>
<td>74.5 ± 10.2</td>
<td>409 ± 38</td>
</tr>
<tr>
<td>C57BL/6 H. felis</td>
<td>182.2 ± 23.2*</td>
<td>654 ± 41*</td>
</tr>
<tr>
<td>μMT mock</td>
<td>54.0 ± 11.9</td>
<td>318 ± 16</td>
</tr>
<tr>
<td>μMT H. felis</td>
<td>125.2 ± 15.8t</td>
<td>670 ± 58t</td>
</tr>
</tbody>
</table>

* Reported as the mean number of BrdUrd positive cells ± SEM per × 20 magnification field at a distance of 1 mm from the forestomach-glandular epithelial junction. Measurements were done by an observer blinded to the experimental protocol.

† Measurements shown represent the mean thickness ± SEM in microns. The gastric mucosal thickness was measured 1 mm from the forestomach-glandular epithelial junction. Measurements were done by an observer blinded to the experimental protocol.

‡ p < 0.05, as compared with mock infected C57BL/6. There were no significant differences when compared with infected μMT.

†† p < 0.05, as compared with mock infected μMT.


