Helicobacter-induced inflammatory bowel disease in IL-10- and T cell-deficient mice

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Burich, Andrew, Robert Hershberg, Kim Waggie, Weiping Zeng, Thea Brabb, Gina Westrich, Joanne L. Viney, and Lillian Maggio-Price. Helicobacter-induced inflammatory bowel disease in IL-10- and T cell-deficient mice. Am J Physiol Gastrointest Liver Physiol 281: G764–G778, 2001.—Inflammatory bowel disease (IBD) is thought to result from a dysregulated mucosal immune response to luminal microbial antigens, with T lymphocytes mediating the colonic pathology. Infection with Helicobacter spp has been reported to cause IBD in immunodeficient mice, some of which lack T lymphocytes. To further understand the role of T cells and microbial antigens in triggering IBD, we infected interleukin (IL)-10−/−, recombinase-activating gene (Rag)1−/−, T-cell receptor (TCR)-α−/−, TCR-β−/−, and wild-type mice with Helicobacter hepaticus or Helicobacter bilis and compared the histopathological IBD phenotype. IL-10−/− mice developed severe diffuse IBD with either H. bilis or H. hepaticus, whereas Rag1−/−, TCR-α−/−, TCR-β−/−, and wild-type mice showed different susceptibilities to Helicobacter spp infection. Proinflammatory cytokine mRNA expression was increased in the colons of Helicobacter-infected IL-10−/− and TCR-α−/− mice with IBD. These results confirm and extend the role of Helicobacter as a useful tool for investigating microbial-induced IBD and show the importance, but not strict dependence, of T cells in the development of bacterial-induced IBD.

Helicobacter bilis; Helicobacter hepaticus; colitis; proinflammatory cytokines

The precise etiology of the human idiopathic inflammatory bowel disorders ulcerative colitis and Crohn’s disease is unknown. Proposed factors include infection with bacterial pathogens, disruption of the intestinal mucosal barrier, dysregulated immune responses to luminal antigens, and genetic susceptibility (2, 35), but their exact roles have not been defined. Within the past decade, numerous mouse models have emerged as tools to investigate mechanisms of initiating and perpetuating inflammatory bowel disease (IBD). Spontaneous intestinal inflammation has been observed in genetically altered mice with various immunologic defects. These include interleukin (IL)-2−/−, IL-10−/−, major histocompatibility complex (MHC) class II−/−, T-cell receptor (TCR) mutants, IL-7 transgenics, and Gα12−/− mice (18, 25, 32, 33, 44). Although the underlying genetic defect that confers susceptibility to IBD varies considerably in these mouse models, a common and central feature is that the development of IBD is dependent on the presence of intestinal luminal flora. For example, IL-2−/− mice maintained in a conventional facility develop IBD, but when rederived into specific pathogen-free (SPF) or germ-free conditions, intestinal lesions are delayed or prevented, respectively (33). Similarly, IL-10−/− mice spontaneously develop IBD when housed under conventional conditions (18), but there is no evidence of colitis when animals are maintained under germ-free conditions (37). In the CD4+ CD45RBhi adoptive transfer model, mice with reduced intestinal flora show less severe wasting disease (1), whereas mice treated with bacitracin and streptomycin are rescued from weight loss (27), again stressing the role that luminal bacteria play in initiating IBD. The adoptive transfer model has also shown that the colitis is mediated by a CD45RBhi subset of CD4+ T cells, (20, 27, 28, 30), highlighting an important role for T cells in the disease process. Few studies have analyzed, in a controlled manner, the relative contribution of bacterial flora and T cell dysregulation in the development of IBD (4).

Infection with murine Helicobacter species has been implicated as a potential cause of “spontaneous” gastrointestinal inflammation mimicking IBD in various mouse strains raised in conventional facilities. Indeed, natural and experimental infections of certain strains of laboratory rodents with particular species of Helicobacter have been shown to cause IBD. For example, immunodeficient mice with natural infections of H. hepaticus exhibit a proliferative typhlocolitis and proctitis (21, 42, 43), and experimental inoculation of severe combined immunodeficiency mice with H. bilis has been reported to result in typhlitis and colitis (14, 39). Recently identified Helicobacter species have also been associated with IBD in IL-10−/− (11) and severe

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combined immunodeficiency mice (15). Although these data by no means suggest that Helicobacter is the only organism capable of initiating murine IBD, they do underscore the importance of this widely endemic bacteria in inducing gastrointestinal inflammation. Therefore, Helicobacter spp may be an excellent model organism with which to investigate the complex role of luminal bacteria in initiating experimental IBD.

Although the above-cited reports have solidified a link between experimental IBD and Helicobacter infection, important questions remain. Given the observation that various Helicobacter species can cause disease, what is the relative efficiency of the different species to induce IBD? Is there identical or disparate pathology? In addition, given the importance of T lymphocytes in experimental IBD, how can one interpret the occurrence of Helicobacter-induced IBD in mice that lack T and B cells (41)? Therefore, the aims of this study were to determine 1) whether H. hepaticus or H. bilis was more potent in producing bowel pathology, 2) whether the IBD induced by Helicobacter spp differs phenotypically in mice with a dysregulated cytokine network compared with mice deficient in specific immune cell subsets, and 3) whether proinflammatory cytokines are regulated during the disease process. To this end, a histopathological scoring system allowed us to quantify the severity and regional extent of disease, and MHC class II expression and cytokine production were analyzed in colonic tissue. Here we report that IL-10−/− mice develop severe IBD after H. bilis or H. hepaticus infection. Furthermore, the IBD that develops is associated with increased MHC class II expression in the intestinal epithelium. None of the C57BL/10J or C57BL/6J immunocompetent mice infected with H. hepaticus or H. bilis showed any evidence of IBD. We also report that mice with absent [recombinase-activating gene (Rag)1−/− mice] or dramatically altered T cell populations [T cell receptor (TCR)-α−/− mice] show mild intestinal inflammation after Helicobacter infection. We analyzed cytokine mRNA expression associated with disease and show that T helper (Th)1-type cytokines are upregulated in the colons of Helicobacter-infected mouse strains with IBD, regardless of whether the model has been previously associated with a Th1 or Th2 cytokine profile.

MATERIALS AND METHODS

Animals. Three- to seven-week-old female C57BL/10J, C57BL/6J, IL-10−/− (C57BL/10J), IL-10−/− (C57BL/6J), Rag1−/− (C57BL/6J), TCR-α−/− (C57BL/6J), and TCR-β−/− (C57BL/6J) mice were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were certified free of Helicobacter spp by the vendor and retested in our animal facilities. Cohorts of mice were housed at the University of Washington (UW) or at Immunex Corporation (IMNX). Animals were housed in a SPF room in polycarbonate microisolator cages containing bed-o’cob (The Andersons, Maumee, OH) and a nestlet. Mice were fed irradiated Picolab rodent diet 20 (PMI Nutrition International, Brentwood, MO) and autoclaved, acidified water. All supplies entering the animal rooms were autoclaved, and the room was maintained at 70–74°F, 45–55% humidity, with 28 air changes per hour and a 12:12-h light-dark cycle. To prevent cross contamination, uninfected and infected mice were housed in separate cubicles or separate rooms. At UW, mice were housed in separate cubicles, depending on infection status, within the same room, and cages were changed in “uninfected” or “infected” laminar flow changing stations. At IMNX, uninfected and infected animals were housed in separate rooms, each containing its own laminar flow changing stations. Sentinel mice at the UW were tested quarterly for endo- and ectoparasites, mouse hepatitis virus, mouse parvovirus, and rotavirus and annually for Mycoplasma pulmonis, pneumonia virus of mice, reovirus-3, Sendai virus, and Theiler’s murine encephalomyelitis virus. Also, colon samples were screened quarterly for Citrobacter rodentium, non-lactose-fermenting Escherichia coli, Salmonella spp, Klebsiella spp, and Clostridium spp (Phoenix Laboratories, Seattle, WA). At IMNX, random sentinel mice from all rooms were tested weekly for mouse hepatitis virus and monthly for 14 mouse viruses (Charles River Laboratories, Wilmington, MA). Biannually, sentinel mice from each room were sent to Charles River Laboratories, necropsied, and screened for several important murine viral, bacteriological, and parasitic pathogens and for Helicobacter spp. The UW Institutional Animal Care and Use Committee and the IMNX Animal Use and Care Committee approved all animal procedures.

Experimental design. Before inoculation, mice were determined to be negative for Helicobacter spp by fecal PCR (see Clinical and histopathological findings associated with Helicobacter spp infection in IL-10−/− mice). In six separate experiments, 5–15 mice were given H. hepaticus or H. bilis, and 3–15 mice were given broth alone. Initially, fecal Helicobacter culture and species-specific fecal PCR were done on pooled cage samples taken every 2 wk until the end of the study. Later experiments used fecal PCR as the sole means to determine Helicobacter infection status. Between 3 and 36 wk postinfection (PI), uninfected and infected mice were euthanized with CO2 and necropsied. Tissues were taken for histopathology, immunohistochemistry, or cytokine analysis or combinations of the three. Mice were weighed weekly and monitored for weight loss, dehydration, and diarrhea. Additionally, fecal samples from both infected and uninfected mice were periodically tested by PCR for cross contamination with another Helicobacter spp in infected mice and to confirm the absence of Helicobacter infection in uninfected animals.

Inoculation. Mice were inoculated by oral gavage with ~2 × 107 H. hepaticus or H. bilis colony-forming units (CFU) in 0.2 ml of Brucella broth or with Brucella broth alone three times within a 9-day period. H. hepaticus was obtained from the American Type Culture Collection (ATCC 51448), and H. bilis was kindly provided by Dr. Lela Riley (University of Missouri, Columbia, MO). Briefly, organisms were streaked onto Brucella blood agar plates (Dept. of Microbiology, Media Laboratory, UW, Seattle, WA), grown in microaerobic conditions (90% N2, 5% H2, and 5% CO2) in vented jars (Oxoid), and kept at 37°C. Bacteria were harvested and inoculated into flasks containing 150 ml of Brucella broth supplemented with 5% FCS (Sigma Chemical, St. Louis, MO). The flasks were placed on a continuous shaker and incubated for 24–48 h at 37°C in microaerobic conditions. The organisms were centrifuged at 10,000 rpm at 4°C for 20 min. The resultant pellet was examined by Gram’s stain and phase microscopy for purity, morphology, and motility (catalase, urease, and oxidase positive). The pellet was resuspended in Brucella broth, and optical density was adjusted to 1.0, as measured at 600 nm, for an estimated 106 CFU/ml (2 × 107 CFU/0.2 ml).
Helicobacter PCR and fecal culture. Feces were analyzed for Helicobacter spp, H. hepaticus, and H. bilis, as described previously (12, 31, 38), with slight modifications. For generic Helicobacter PCR, 5 µl of unquantitated fecal DNA were used as a template, and for H. hepaticus and H. bilis PCR, 10 µl of unquantitated fecal DNA were used as a template for all samples. Primers, their sequences, and the PCR conditions used are summarized in Table 1. To correlate fecal culture results and PCR status, fecal pellets were vortexed in PBS and incubated at room temperature for 30 min. Fecal slurries were then filtered through an 0.8-µm filter, and the filtrate was streaked onto Brucella blood agar plates containing trimethoprim, vancomycin, and polymyxin and incubated as described in Inoculation. Cultures were maintained for 2–3 wk before determination of negative growth was made.

Pathology. The colon was fixed in 10% buffered formalin with a “Swiss roll” technique (26). The liver, cecum, rectum, and mesenteric lymph nodes were also fixed in formalin. Fixed tissues were routinely processed, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. Tissue sections were coded to blind the pathologist (K. Waggie) to the strain and infection status of the animal. The cecum, proximal colon, middle colon, distal colon, and rectum from each mouse were scored on severity of mucosal epithelial changes, degree of inflammation, and extent of pathology (Table 2). The segment score was derived by summing the severity scores [segment score = mucosal score + inflammation score + extent of segment affected in any manner (extent 1) + extent of segment affected at level 3 or 4 (extent 2); maximum segment score was 15]. The total score for each mouse was derived by summing the scores from the individual segments (maximum total score was 75). Subsequently, the mean segment and total scores were derived for each treatment group.

Table 1. Conditions for PCR reactions

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Helicobacter Genus Specific</th>
<th>PCR Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>H276f</td>
<td>CTA TGA CGG GTT ACC TTT</td>
<td>PCR mixtures were heated at 94°C for 5 min, followed by 35 cycles, each comprising 45 sec at 94°C, 1 min at 53°C, 2 min at 72°C, and final extension at 72°C for 8 min</td>
<td></td>
</tr>
<tr>
<td>H676r</td>
<td>GGT ATT GCA TCT CTT</td>
<td>PCR mixtures were heated at 94°C for 5 min, cooled to 61°C; at this time 3 U of Amplitherm DNA polymerase and 5 µl of enhancer were added, followed by 35 cycles, each comprising 1 min at 94°C, 2.25 min at 61°C, 2.5 min at 72°C, and final extension at 72°C for 7 min</td>
<td></td>
</tr>
<tr>
<td>B39p</td>
<td>CTG TTT TCA AGC TCC</td>
<td>PCR mixtures were heated at 94°C for 5 min, cooled to 61°C; at this time 3 U of Amplitherm DNA polymerase and 5 µl of enhancer were added, followed by 35 cycles, each comprising 1 min at 94°C, 1.5 min at 61°C, 2 min at 72°C, and final extension at 72°C for 5 min 40 s</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Scoring system for histological evaluation of IBD lesions

<table>
<thead>
<tr>
<th>Mucosa</th>
<th>Inflammation</th>
<th>Extent*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 No significant lesions</td>
<td>0 None</td>
<td>0 No significant changes</td>
</tr>
<tr>
<td>1 Mild epithelial hyperplasia</td>
<td>1 Mild inflammation limited to mucosa</td>
<td>1 &lt;1% of segment affected</td>
</tr>
<tr>
<td>2 Moderate epithelial hyperplasia</td>
<td>2 Moderate inflammation in mucosa and submucosa</td>
<td>2 1–30% of segment affected</td>
</tr>
<tr>
<td>3 Severe epithelial hyperplasia with crypt branching or herniation</td>
<td>3 Severe inflammation with obliteration of normal architecture, erosions, and/or crypts abscessed</td>
<td>3 1–60% of segment affected</td>
</tr>
<tr>
<td></td>
<td>4 Level 3 changes + ulceration</td>
<td>4 &gt;60% of segment affected</td>
</tr>
</tbody>
</table>

M, mucosa; I, inflammation; E, extent; IBD, inflammatory bowel disease. Nos. at left represent lesion severity scores. Segment score = M + I + E1 + E2. *E1 = %segment affected in any manner; E2 = %segment with level 3 or 4 changes.
2-mercaptoethanol (Life Technologies, Rockville, MD). RNA was isolated over a cesium chloride cushion and subjected to phenol chloroform extraction as previously described (34). cDNA was prepared from 5 μg of isolated RNA with Super-Script II reverse transcriptase (Life Technologies, Rockville, MD) in the presence of 0.3 μg of random primers, 5 × RT buffer, 0.1 M dithiothreitol, 10 mM deoxynucleotide triphosphate, and water. Interferon-γ and IL-4 messages were analyzed by RT-PCR with the mouse TH1/TH2 Switch Cytokine kit (BioSource International, Camarillo, CA). The following profile was used for amplification: 96°C for 1 min followed by 2 cycles of 96°C for 1 min and 59°C for 4 min, then 30 cycles of 94°C for 1 min and 59°C for 2.5 min, and a final cycle of 70°C for 10 min. As a negative control, cDNA was replaced with water, and the sample was subjected to the same PCR conditions. The PCR product and 1 μl of loading buffer were loaded on a 1.0-mm, 15-well, 10% TBE precast gel (Novex, San Diego, CA) and run at 126 V. The gel was stained with ethidium bromide and photographed. For RNase protection assay analysis, Helicobacter bilis-induced IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1RA) levels were measured at selected time points in IL-10−/−, C57BL/6J, and Rag1−/− mice with the RiboQuant Multiprobe RNase Protection Assay kit mCK-2b (PharMingen). The assay was run according to the manufacturer’s instructions, and the values obtained for each sample were normalized to glyceraldehyde-3-phosphate dehydrogenase.

Statistical analyses. Differences between mean total pathology scores in IL-10−/− mice infected with either Helicobacter bilis or H. hepaticus were evaluated by two-way analysis of variance (Sigma Stat 2.0; Jandel, San Rafael, CA). Statistical significance was set at a P value <0.05.

RESULTS

Colonization with Helicobacter hepaticus or H. bilis. Mice inoculated with Helicobacter hepaticus or H. bilis became fecal PCR positive 2–4 wk postinoculation and remained persistently positive for the duration of these experiments (12–36 wk PI). Consistent with other studies, PCR was a more sensitive and specific means to detect Helicobacter spp infection compared with culture (data not shown) (22). Therefore, PCR was the sole method used to confirm infection. Uninfected animals that received broth alone remained Helicobacter fecal PCR negative throughout these studies, as determined with Helicobacter genus primers. Periodic fecal PCR for the alternate Helicobacter spp with species-specific primers revealed that at no time were the mice cross contaminated.

Clinical and histopathological findings associated with Helicobacter spp infection in IL-10−/− mice. An initial study (study 1) was performed to determine whether different species of Helicobacter induced similar or dissimilar forms of IBD after infection with either Helicobacter bilis or H. hepaticus in a well-characterized IBD model, the IL-10−/− mouse. Diarrhea was evident in the Helicobacter spp-infected IL-10−/− mice, but there were notable differences between the clinical course of Helicobacter bilis and H. hepaticus-infected animals. The earliest time to development of diarrhea differed depending on whether IL-10−/− mice were infected with H. hepaticus (1.5 wk) or H. bilis (3 wk). Despite the more rapid induction of diarrhea with Helicobacter hepaticus, weight gain in Helicobacter bilis-infected IL-10−/− mice was less than that seen in uninfected controls (Fig. 1A), whereas Helicobacter hepaticus-infected IL-10−/− mice showed normal growth curves (data not shown). It is important to note that there were no significant weight change differences in Helicobacter bilis-infected wild-type mice (Fig. 1B). Diarrhea was not observed in Helicobacter bilis-infected wild-type mice or in uninfected wild-type and IL-10−/− mice.

Both Helicobacter bilis and Helicobacter hepaticus induced IBD in IL-10−/− mice (Fig. 2B and data not shown), whereas no IBD was observed in uninfected IL-10−/− mice (Fig. 2A) or Helicobacter-infected and uninfected wild-type C57BL/10J mice (Fig. 2C). Generally, both Helicobacter bilis and H. hepaticus produced chronic proliferative typhlocolitis and proctitis beginning at 3 wk PI. The mean total pathology scores are summarized in Table 3.

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Although *H. hepaticus* produced its most severe lesions in the cecum, proximal colon, and rectum, *H. bilis* induced significant disease in all portions of the large bowel.

With *H. hepaticus* infection, the chronic colitis was characterized by varying degrees of mucosal thickening resulting from epithelial hyperplasia, with elongation and occasional branching of crypts (data not shown). Crypt penetration into the submucosa was observed in the rectum of two of three mice 12 wk PI (data not shown). An inflammatory cell infiltrate was present that consisted primarily of lymphocytes and macrophages with varying numbers of plasma cells and neutrophils. The infiltrate was most often confined to the lamina propria and submucosa but was occasionally transmural. Crypt abscesses and mucosal erosions...
were infrequently present. The typhlitis was characterized by severe mucosal hyperplasia and scattered erosions, goblet cell loss, and a predominantly lymphoplasmacytic infiltrate (Fig. 3, A and B).

Microscopic changes in *H. bilis*-infected IL-10−/− mice were similar to those observed in *H. hepaticus* animals but tended to be more severe, especially in the middle and distal colon 7 and 12 wk PI (Table 3). Specifically, severe dilation of the submucosal lymphatics and submucosal edema were found at 3 and 7 wk PI. Transmural inflammation, crypt abscesses, and mucosal erosions were frequently observed at all time points, which differed from findings with *H. hepaticus* infection. Crypt penetration into the submucosa, observed at 12 wk PI in two of three mice, extended into the distal colon of *H. bilis*-infected mice (Fig. 2B), and the rectum was similarly affected (Fig. 3, C and D).

**Table 3. Pathology scores in IL-10−/− mice infected with *H. hepaticus* or *H. bilis***

<table>
<thead>
<tr>
<th>Infection Status</th>
<th>Wk PI</th>
<th>Cecum</th>
<th>Proximal Colon</th>
<th>Middle Colon</th>
<th>Distal Colon</th>
<th>Rectum</th>
<th>Total Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. hepaticus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>11</td>
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<td>11</td>
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<td>12</td>
<td>10</td>
<td>7</td>
<td>6</td>
<td>11</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td><em>H. bilis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>11</td>
<td>12</td>
<td>12</td>
<td>58</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means derived from 2–3 mice (study 1) and 15 mice (study 2). Maximum segment score is 15, and maximum total score is 75. Total mean scores may not always equal the sum of segment mean scores because of rounding error. *H.*, *Helicobacter*; IL, interleukin; PI, postinfection. In study 1, uninfected control IL-10−/− mice did not develop any evidence of IBD during the course of this study. In study 2, evidence of spontaneous IBD was observed in 4 of 15 control IL-10−/− mice (data not shown).

Fig. 3. IL-10−/− mice infected with *H. hepaticus* or *H. bilis* developed proliferative typhlitis and proctitis beginning 3 wk PI. A: cecum of uninfected IL-10−/− mouse 12 wk after treatment. B: cecum of IL-10−/− mouse infected with *H. hepaticus* 12 wk PI. Note severe mucosal hyperplasia, inflammation, and focal erosion. Lumens of occasional crypts are dilated and contain debris. Ceca of *H. bilis*-infected mice at this time point had a similar appearance. C: rectum of uninfected IL-10−/− mouse 12 wk after treatment. D: rectum of IL-10−/− infected with *H. bilis* 12 wk PI. Note crypt branching and herniation. Similar lesions were present at 3 and 7 wk PI. Segments stained with hematoxylin and eosin; original magnification, ×10.
The staining was evident on both epithelial cells and cells within the lamina propria (Fig. 4A), with scores ranging from 2 to 3 (Table 4). Uninfected IL-10-/- mice without colitis showed a minimal to moderate increase in MHC class II expression (Fig. 4B; Table 4). H. hepaticus- and H. bilis-infected wild-type mice had minimal MHC class II expression that was primarily localized to the surface colonic epithelial cells (Fig. 4C). As a scoring reference, colonic tissue from MHC II-/- mice was stained and showed no MHC class II expression (Fig. 4D).

Our initial observations suggested that H. bilis infection in IL-10-/- mice may produce more extensive and severe IBD than H. hepaticus (Table 3). Therefore, in study 2, a larger group of IL-10-/- mice were infected with H. bilis or H. hepaticus to determine whether this difference was statistically significant. It was necessary to euthanize all mice at 4 wk PI because of weight loss observed in the IL-10-/- mice infected with H. bilis. This study, consisting of mixed ages of mice, showed no statistically significant differences between H. bilis- and H. hepaticus-induced IBD pathology (Fig. 5, A and B). Mean total pathology scores were 43 and 44 for IL-10-/- mice infected with H. bilis or H. hepaticus, respectively (P = 0.605; Table 3). A low level of mild spontaneous IBD (mean total pathology score of 2) was observed in 4 of 15 uninfected IL-10-/- mice (data not shown), whereas all others remained disease free (Fig. 5C). Interestingly, when mean total pathology scores from mice that were older (6–7 wk of age; data not shown) at the time of Helicobacter infection were analyzed and compared, we found a trend towards significance (P = 0.076). Based on this observation, as well as the altered growth curves seen in H. bilis-infected compared with H. hepaticus-infected IL-10-/- mice, it is our impression that H. bilis-induced IBD in IL-10-/- mice tends to be more severe than H. hepaticus-induced disease, especially in mice that are older at the time of Helicobacter infection. Clearly, the data support the hypothesis that both species of Helicobacter can induce severe IBD in this model.
Table 4. MHC class II expression in proximal and distal colon of Helicobacter spp-infected and -uninfected mice

<table>
<thead>
<tr>
<th>Mouse Strain, Infection Status</th>
<th>3 Wk PI Proximal</th>
<th>3 Wk PI Distal</th>
<th>7 Wk PI Proximal</th>
<th>7 Wk PI Distal</th>
<th>12 Wk PI Proximal</th>
<th>12 Wk PI Distal</th>
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<tbody>
<tr>
<td>Rag1^{-/-}</td>
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<td>1</td>
<td>2</td>
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<tr>
<td><em>H. hepaticus</em></td>
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<td>2</td>
<td>2</td>
<td>2</td>
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<td>1</td>
</tr>
<tr>
<td><em>H. bilis</em></td>
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<td>1</td>
<td>1.5</td>
<td>1.5</td>
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<td>1</td>
</tr>
<tr>
<td>Broth</td>
<td>2.7</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><em>H. hepaticus</em></td>
<td>2.3</td>
<td>2.7</td>
<td>3</td>
<td>2.3</td>
<td>2</td>
<td>2.7</td>
</tr>
<tr>
<td>Broth</td>
<td>1</td>
<td>1.5</td>
<td>1.5</td>
<td>1</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td>C57BL/10J</td>
<td>1</td>
<td>1</td>
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Values are means derived from 2 or 3 animals; for reference, colonic scores from MHC class II^{-/-} mice were 0. Scores ranged from 0 (no expression) to 3 (maximal expression). MHC, major histocompatibility complex; Rag, recombining-activating gene. Numbers reflect a mean derived from 2 or 3 animals.

Susceptibility of Rag1^{-/-} mice to Helicobacter spp infection. Rag1^{-/-} mice infected with either *H. hepaticus* or *H. bilis* did not develop the clinical or typical histological features of IBD that were seen in the IL-10^{-/-} mice in studies extending to 12 wk PI. Similarly, uninfected Rag1^{-/-} mice showed no evidence of intestinal inflammation. However, one of three mice from the *H. hepaticus* and *H. bilis* groups developed an acute, focal, mild inflammation of the colon and/or cecum at the 3 or 12 wk time points, respectively. Although *Helicobacter* spp-infected Rag1^{-/-} mice did not develop IBD, MHC class II expression was moderately increased (score of 2) in the proximal colon of *H. hepaticus*-infected Rag1^{-/-} mice at 3 and 7 wk PI (Table 4) compared with uninfected controls. Similarly, *H. bilis*-infected Rag1^{-/-} mice showed mild to moderate (score of 1.3–2.0) MHC class II staining in the proximal and distal colon at 3 and 7 wk PI. At all time points examined, uninfected Rag1^{-/-} mice showed mild MHC class II staining intensity of the proximal and distal colon (score of 1.0–1.5; Table 4).

To determine whether long-term infection with *Helicobacter* could initiate IBD in Rag1^{-/-} mice, both wild-type and Rag1^{-/-} mice were infected with either *H. hepaticus* or *H. bilis* and their progress was followed for 30–36 wk. Significant morbidity occurred in our Rag1^{-/-} mice as a result of *Pneumocystis carinii* infection and precipitated the end of the study at these time points. Approximately 9 mo after *Helicobacter* infection, there were no clinical indications of IBD (diarrhea, rectal prolapse) in Rag1^{-/-} or wild-type mice chronically infected with *Helicobacter*. This is in marked contrast to the rapid onset of disease in similarly infected IL-10^{-/-} mice (1.5–3 wk PI). Additionally, no histological evidence of IBD was found in *Helicobacter*-infected and uninfected wild-type mice or uninfected Rag1^{-/-} mice (Fig. 6, A and C; data not shown). However, two of five Rag1^{-/-} mice in both the *H. hepaticus* and *H. bilis* groups developed mild focal mucosal inflammation characterized by minimal to mild epithelial hyperplasia and low numbers of a predominantly neutrophilic infiltrate (Table 5; Fig. 6, B and D). Interestingly, lesions in *H. bilis*-infected Rag1^{-/-} mice were found solely in the proximal colon, whereas those of *H. hepaticus*-infected Rag1^{-/-} mice were found in the cecum (Fig. 6, B and D). It is important to note that typical IBD lesions such as those seen in similarly infected IL-10^{-/-} mice did not develop in *Helicobacter*-infected Rag1^{-/-} mice.

Susceptibility of T cell-deficient mice to *H. bilis* infection. Because we were unable to induce significant IBD with *Helicobacter* spp in a T cell-deficient mouse (Rag1^{-/-}) but did obtain severe intestinal inflammation with *Helicobacter* in a model having T cells (IL-10^{-/-}), we wanted to determine the importance of T cell populations in the initiation of IBD. Therefore, we infected wild-type, TCR-α^{-/-}, and TCR-β^{-/-} mice with *H. bilis* in the same manner previously described. We chose *H. bilis* for these experiments based on our clinical experience with this organism in eliciting profound rapid inflammation in the IL-10^{-/-} mouse. Two separate experiments were performed, extending to 17.5 (study 1) and 29 (study 2) wk PI. *Helicobacter*-infected TCR-β^{-/-} mice were only included in the initial 17.5-wk study. Of these two mutant strains, we were only able to induce IBD in TCR-α^{-/-} mice in the time frame of these studies. The onset of disease in the TCR-α^{-/-} mice was more delayed than that historically seen in the IL-10^{-/-} mice infected under a similar protocol. Clinically, diarrhea was evident in the TCR-α^{-/-} mice in these two studies beginning 10–12.5 wk PI. However, diarrhea was not evident in all *Helicobacter*-infected TCR-α^{-/-} mice. This was much later than what we had observed in *H. bilis*-infected IL-10^{-/-} mice (3 wk PI). Furthermore, growth curves for *H. bilis*-infected and uninfected TCR-α^{-/-} mice were similar (data not shown).

At 17.5 wk PI, three of five *H. bilis*-infected TCR-α^{-/-} mice had lesions in the cecum, colon, and rectum that were similar to but milder than lesions observed in the corresponding *H. bilis*-infected IL-10^{-/-} mice (see Clinical and histopathological findings associated...
with *Helicobacter* spp infection in IL-10−/− mice). Pathology scores are summarized in Table 5. Although lesions in the TCR-α−/− mice were found in all areas of the large bowel, they were segmental within each area. Generally, the lesions were characterized by moderate crypt epithelial hyperplasia with inflammatory cell infiltrates limited to the mucosa and submucosa (Fig. 7). One of four uninfected TCR-α−/− and one of four uninfected TCR-β−/− mice had mild colitis limited to a focal area in the middle colon. These focal lesions were similar to but milder than those in the *H. bilis*-infected TCR-α−/− mice and were probably the result of the non-*Helicobacter*-associated colitis reported to occur spontaneously in these strains (25).

Study 2 was initiated to correlate histopathology and cytokine regulation during *Helicobacter*-induced IBD. After 4 weeks of infection, pathology scores were again evaluated (Table 5). The severity of lesions in TCR-α−/− mice was not significantly different from those in TCR-β−/− mice. A: distal colon of IL-10−/− mouse infected with *H. bilis* 4 wk PI. Note severe epithelial hyperplasia with a mixed inflammatory cell infiltrate into the lamina propria, extending somewhat into the submucosa. B: distal colon of IL-10−/− mouse infected with *H. hepaticus* 4 wk PI. Disease is similar to that seen in IL-10−/− mice infected with *H. bilis*, with severe epithelial hyperplasia and mucosal inflammation. However, rarely did the inflammatory cells infiltrate the submucosa. C: distal colon of uninfected IL-10−/− mouse 4 wk after treatment. This uninfected mouse shows no evidence of inflammation, although 4 of 15 uninfected IL-10−/− mice did show very mild spontaneous IBD. Segments stained with hematoxylin and eosin; original magnification, ×10.
IBD in TCR-α−/− mice (see Proinflammatory cytokine production in the colon of H. bilis-infected mice for cytokine results). In mice evaluated histologically at 29 wk PI, four of eight H. bilis-infected TCR-α−/− mice showed IBD lesions similar to those seen in our initial study (Table 5). Interestingly, the IBD in these diseased TCR-α−/− mice was completely subclinical, with no evidence of diarrhea or weight loss (data not shown). Again, we saw evidence of mild, focal, spontaneous IBD in uninfected TCR-α−/− mice (2 of 8).

Proinflammatory cytokine production in the colon of H. bilis-infected mice. Proinflammatory cytokine expression correlated with the presence of intestinal inflammation in IL-10−/− and TCR-α−/− mice. With RT-PCR, IL-10−/− mice infected with H. bilis showed...
elevated levels of interferon-γ mRNA in the colon compared with uninfected IL-10−/− mice at all time points analyzed (Fig. 8A). Our results agree with previous studies that reported interferon-γ release on stimulation of mesenteric lymph node cells from *H. hepaticus*-infected IL-10−/− mice (19). Surprisingly, elevated levels of interferon-γ message were also seen in colonic tissue harvested from diseased *H. bilis*-infected TCR-α−/− mice compared with uninfected TCR-α−/− mice (Fig. 8B). Although this model is recognized as a predominantly IL-4-driven model of IBD, we did not see elevated levels of IL-4 message in either *H. bilis*-infected or uninfected TCR-α−/− mice. In contrast, colonic tissue samples from *H. bilis*-infected Rag1−/−, TCR-β−/−, and wild-type mice (Fig. 8A and data not shown) showed greatly reduced amounts of interferon-γ message compared with *H. bilis*-infected IL-10−/− mice with IBD. Using an RNase protection assay, we found that *H. bilis* upregulated expression of IL-1α (~3-fold), IL-1β (~15- to 26-fold), and IL-1RA (~3-fold) in IL-10−/− mice with IBD (Fig. 9). As an indicator of dysregulation of proinflammatory cytokine activity, the IL-1RA-to-IL-1β ratio was determined for infected versus uninfected IL-10−/− mice. *H. bilis*-infected IL-10−/− mice had a lower ratio (0.626) than uninfected IL-10−/− mice (5.921). Expression of IL-1α,
Fig. 8. *H. bilis* induced increased mRNA expression of interferon (IFN)-γ in the colon of IL-10−/− and TCR-α−/− mice with IBD analyzed by RT-PCR. A: low levels of IFN-γ are seen in IL-10−/− uninfected, *H. bilis*-infected C57BL/6J, and *H. bilis*-infected and uninfected Rag1−/− mice. Additionally, low levels of IL-4 were seen in both *H. bilis*-infected and uninfected Rag1−/− mice. RNA samples (3–5 sets) were prepared from each infected and uninfected strain 6–17 wk PI. For each sample, the PCR reaction was performed 1–3 times. IL-10−/− mice were 6 wk PI, whereas the C57BL/6J and Rag1−/− mice were 17 wk PI. B: IFN-γ levels were significantly elevated in diseased *H. bilis*-infected TCR-α−/− mice compared with uninfected controls. Note the absence of IL-4 message in diseased TCR-α−/− mice. RNA samples were prepared from infected and uninfected TCR-α−/− mice 13 wk PI, and the PCR reaction was performed twice on each sample. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 9. *H. bilis* induced increased mRNA expression of IL-1α (B), IL-1β (C), and IL-1 receptor antagonist (RA; D) in the colon of IL-10−/− mice as detected by RNase protection assay. No such elevations were seen in *H. bilis*-infected C57BL/6J mice (A–D). IL-10−/− and wild-type mice were 9 and 7 wk post-*H. bilis* infection, respectively. All values are normalized to GAPDH.
IL-1β, and IL-1RA in infected and uninfected wild-type and Rag1−/− mice was not significantly different (data not shown).

DISCUSSION

Previous studies (9, 18, 29, 33, 37) have shown that luminal bacteria are an important factor contributing to the development of IBD in mice and humans. Several laboratories have implicated infection with Helicobacter species in the development of mucosal inflammation and IBD in various mouse models (4, 6, 13, 14, 39). By comparing the effects of the less widely studied H. bilis with H. hepaticus in the IL-10−/− mouse, a well-established model of Th1-driven IBD (3, 7), we present data that both confirm and extend these results that suggest a role for Helicobacter in murine IBD. In addition, because of emerging literature suggesting that Helicobacter-induced IBD can occur in profoundly immunodeficient mice, we attempted to address the role of T cells in the pathogenesis of Helicobacter-induced IBD. Using the combination of a detailed histopathological scoring system, MHC class II immunohistochemistry, and cytokine analysis, we demonstrate herein that both H. bilis and H. hepaticus consistently produce severe IBD in IL-10−/− mice, whereas the ability of Helicobacter to induce intestinal inflammation in mice with absent T cells (Rag1−/− and TCR-β−/− mice) or significantly altered T cell subsets (TCR-α−/− mice) is attenuated. Furthermore, increases in MHC class II expression and colonic proinflammatory cytokine levels correlated with the presence of IBD in Helicobacter-infected IL-10−/− mice. Surprisingly, despite the fact that the TCR-α−/− mouse has been described as a “Th2” model of murine IBD (23, 24, 40), Helicobacter infection of these mice resulted in colitis with elevated levels of interferon-γ without significant induction of IL-4. Taken together, the current studies strongly support the role of murine Helicobacter in the development of colitis and highlight the importance of, but not strict dependence on, T cells in the development of bacterially driven mucosal inflammation in mouse strains genetically susceptible to the development of IBD.

Numerous studies have investigated Helicobacter spp as model organisms in microbial-induced IBD. It is fair to say that the observations made in these studies have varied widely. For example, at one end of the spectrum, Kullberg et al. (19) have observed chronic colitis in SPF-reared IL-10−/− mice after infection with H. hepaticus. In an independent series of experiments, Chin et al. (6) described the ability of H. hepaticus to induce significant mucosal inflammation in the cecum and colon of TCR-αβ mutant mice. In marked contrast, recent studies have reported that Helicobacter does not induce IBD in germ-free IL-10−/− mice or potentiate IBD in germ-free IL-10−/− mice that have been reconstituted with SPF flora (8, 37). It is important to note that the published studies have used different protocols in varied mouse facilities and, in so doing, have not resulted in a consensus regarding the role of Helicobacter as a potential pathogen in conventional mouse facilities or its utility as a model organism to study IBD. How do the data presented in this report fit into the context of these previous studies? Clearly, we have shown that Helicobacter can be an important determinant in the induction of murine colitis in an SPF mouse facility. In our experience, IBD-prone strains such as IL-10−/− and TCR-α−/− mice rarely develop mucosal inflammation in a Helicobacter-free, SPF environment, whereas monoinfection with H. bilis or H. hepaticus induces a significantly greater incidence and severity of disease in these mice. A further conclusion from these studies is that the disease induced by Helicobacter varied significantly among the different IBD models. Foremost, both H. bilis and H. hepaticus reliably induced severe chronic IBD in the IL-10−/− mouse model of IBD. Disease was evident clinically 1.5–3 wk PI, and inflammation was evident from cecum to rectum. Intermediate in disease severity was the intestinal inflammation found in our TCR-α−/− mice infected with H. bilis. Lesions were similar to but milder than the inflammation seen in similarly infected IL-10−/− mice. Additionally, disease incidence in the Helicobacter-infected TCR-α−/− mice was less than that seen in Helicobacter-infected IL-10−/− mice. In contrast, minimal mucosal inflammation was found in Rag1−/− mice, which do not have T or B cells. This inflammation, when present, was characterized by minimal to mild focal epithelial hyperplasia and had a primarily neutrophilic infiltrate. Our observations support those of others that show Helicobacter can induce fulminant disease in certain immunodeficient strains of mice.

As mentioned, there are important differences among all these studies that may explain the varied results. In our opinion, the major variables include the unique bacterial flora present in geographically different mouse colonies and the contribution of genetic background to IBD susceptibility. Regarding the importance of mouse strain variation in the development of IBD, Berg et al. (3) have shown that IL-10−/− mice on a 129/SvEv or BALB/c background are inherently more susceptible than IL-10−/− mice on a C57BL/6J background. Strain differences may explain why in our studies with Helicobacter-infected TCR-α−/− and TCR-β−/− mice, we saw less severe disease than that reported by Chin et al. (6). Specifically, our mice were on a C57BL/6J background, whereas those of Chin et al. were on a C57BL/6 × 129-Ola background. Moreover, it is nearly impossible to characterize the subtle yet potentially important differences in intestinal microbial flora between different colonies of mice. We and others (8) assume that in the complex ecosystem of the colon microbiota, Helicobacter alone is not sufficient (even in a highly susceptible model like the IL-10−/− mouse) to induce mucosal inflammation. This may explain the findings that germ-free IL-10−/− mice infected with H. hepaticus do not develop IBD (8). Therefore, the profound inflammation seen in germ-free mice reconstituted with SPF flora, with or without Helicobacter (8, 37), does not discount the potential role of Helicobacter in murine...
colitis. Instead, in our opinion, it reinforces the hypothesis that multiple bacterial species can contribute to the establishment of IBD, particularly in a setting where mice without natural tolerance to luminal bacterial (as in germ-free mice not exposed to normal flora during development) are exposed to a wide range of bacterial flora.

Numerous studies have underlined a prominent role for T cells in the initiation and maintenance of IBD (3, 7, 17, 25, 28, 30). Therefore, it is somewhat surprising that several studies have suggested that Helicobacter can induce IBD in animal models devoid of T cells (6, 14, 15, 21, 39, 41). We tested this question directly by infecting Rag1⁻/⁻ mice, which lack T and B cells, with H. bilis or H. hepaticus. In contrast to the consistent induction of severe IBD in IL-10⁻/⁻ mice infected with Helicobacter spp, we observed mild acute inflammation in Rag1⁻/⁻ mice. It is also important to note that the mucosal inflammation observed in Helicobacter-infected Rag1⁻/⁻ mice was more focal than that seen in similarly infected IL-10⁻/⁻ and TCR-α⁻/⁻ mice and was particularly less consistent in its appearance. Still, the intestinal inflammation seen in Rag1⁻/⁻ mice infected with Helicobacter suggests that the development of bacterial-driven mucosal inflammation is not strictly dependent on T cells. It is tempting to speculate that Helicobacter may trigger the innate immune system resulting in the induction of cytokines and/or other chemical mediators that may eventually produce inflammation. Whether it is through the modulation of intestinal barrier function and/or induction of proinflammatory cytokines via lipopolysaccharide-mediated activation of Toll-like receptors on dendritic cells, monocytes, or epithelial cells or via recently discovered virulence factors in certain Helicobacter spp (5, 45), this innate immune response may contribute to the establishment of mucosal inflammation and IBD.

In summary, our results in a variety of mouse models suggest that Helicobacter infection plays an important role in the induction of IBD in SPF mice. In our experience, T cells appear to be required for the development of chronic, diffuse, and severe inflammation associated with Helicobacter infection. Still, the observations in Rag1⁻/⁻ mice presented here, along with a recent report from another group (6), demonstrate that T cells are not strictly required for the induction of mucosal inflammation by Helicobacter. In addition, we show that Helicobacter may harbor the ability to skew the cytokine responses toward a Th1 phenotype, as shown by the elevated levels of interferon-γ in the colons of Helicobacter-infected mice. Again, we suggest that Helicobacter is likely to be one of a group of bacteria that, in mice, can tip the balance toward mucosal inflammation. Whether there are unifying and/or overlapping features between these microbial triggers for IBD (predilection for skewing toward Th1 responses, for example) remains to be determined. However, a recent report has shown the ability of Citrobacter rodentium to elicit a Th1 cytokine phenotype associated with IBD, suggesting that Th1 responses may be a stereotypical response to certain luminal microbes (16). We believe organisms like Helicobacter can serve as useful tools to study microbial-induced IBD and may be able to both assist in refining the hypotheses regarding the molecular pathogenesis of IBD and to test novel therapeutic interventions that may be relevant to the treatment of human IBD. Although there are currently no data to suggest that Helicobacter spp are implicated in human Crohn’s disease or ulcerative colitis, we are intrigued by recent observations of novel Helicobacter species in both the cotton-top tamarin (36) and in humans with diarrhea (10).

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REFERENCES


