A Novel Urease-Negative Helicobacter Species Associated with Colitis and Typhlitis in IL-10-Deficient Mice

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A spiral-shaped bacterium with bipolar, single-sheathed flagella was isolated from the intestines of IL-10 (interleukin-10)-deficient (IL-10−/−) mice with inflammatory bowel disease. The organism was microaerobic, grew at 37 and 42°C, and was oxidase and catalase positive but urease negative. On the basis of 16S rRNA gene sequence analysis and biochemical and phenotypic criteria, the organism is classified as a novel helicobacter, Helicobacter hepaticus. Cesarean section-rederived IL-10−/− mice without helicobacter infection did not have histological evidence of intestinal inflammation. However, helicobacter-free IL-10−/−, SCID/NCr, and A/JNCr mice experimentally inoculated with the novel urease-negative Helicobacter sp. developed variable degrees of inflammation in the lower intestine, and in immunocompetent mice, the experimental infection was accompanied by a corresponding elevated immunoglobulin G antibody response to the novel Helicobacter sp. antigen. These data support other recent studies which demonstrate that multiple Helicobacter spp. in both naturally and experimentally infected mice can induce inflammatory bowel disease. The mouse model of helicobacter-associated intestinal inflammation should prove valuable in understanding how specific microbial antigens influence a complex disease process.

The type species of the genus Helicobacter, H. pylori, is known to cause a persistent inflammatory response in the human stomach and in some cases is directly linked to peptic ulcer disease and the development of gastric cancer (8, 13, 15). In 1994, a novel helicobacter, H. hepaticus, was isolated from the livers of A/JCr mice with a high incidence of chronic hepatitis and hepatocellular carcinoma (5, 27). Coincident with isolation of H. hepaticus from livers of infected mice, the organism was cultured from intestinal crypts of the colon and cecum (5). Shortly thereafter, we isolated H. hepaticus from inflamed lower bowel tissue of some immunodeficient strains of mice (athymic NCr-nu, BALB/c AnNcr-nu, C57BL/6 NCr-nu, and SCID/NCr) with chronic proliferative colitis and proctitis (26). When experimentally inoculated into either germfree outbred mice, defined-flora SCID mice, or specific-pathogen-free (SPF) A/JCr mice with normal microbial flora, H. hepaticus causes variable degrees of persistent inflammation of the colons and cecum of infected mice (2, 7, 28).

Inflammatory bowel disease (IBD) has also been recognized in lines of mice genetically deficient in production of the cytokines (IL-2 [interleukin-2] and IL-10) and those lacking T-cell receptor (TcR) α chain and TcR β chain (10, 14, 17). IBD is hypothesized to result from a combination of genetic and environmental factors. It is not clear whether the aberrant mucosal immune response seen in IBD is the result of a response to the microbial biota of the gastrointestinal tract or if the damage results from an immune response to self antigens (Ags). The theory favoring microbial Ags as inducing this response, in part, was substantiated in IL-10-deficient (IL-10−/−), IL-2−/−, and TcRα−/− mice, whose clinical and histologic presentation of IBD was attenuated when the mice were maintained under SPF conditions (10). In addition, IL-2−/− mice raised under germfree conditions failed to develop IBD (17). Furthermore, IL-10−/− and TcRα−/− helicobacter-free mice experimentally infected with H. hepaticus develop IBD, whereas controls do not (3, 11).

In this report we describe the isolation of a novel urease-negative Helicobacter sp. from IL-10−/− mice with IBD and document the prevention of intestinal inflammation in helicobacter-free IL-10−/− cesarean-derived mice. We also demonstrate the induction of lower bowel inflammation in selected strains of mice experimentally inoculated with this novel Helicobacter sp.

MATERIALS AND METHODS

Animals. IL-10−/− mice on a C57BL6/129-Ola background were provided by R. Kuhn and W. Müller (10). These animals were initially housed in conventional animal facilities. Clinically, the mice had a high incidence of rectal prolapse, and most of them died by 4 months of age.

Bacterial isolation. Two of the original non-SPF IL-10−/− knockout mice, one with and one without rectal prolapse, were euthanatized with CO2. Cecal contents and feces were collected from each mouse and resuspended in brain heart infusion broth with horse serum and yeast extract; the slurry was passed through a 0.45-μm-pore size filter with a stacked prefiter. The filtered fecal material was then inoculated onto brucella sheep blood agar with trimethoprim, vancomycin, and polymycin (Remel Laboratories, Lenexa, Kan.) and incubated at 37 or 42°C under microaerobic conditions for up to 5 days. Bacterial isolates were tested for oxidase, catalase, and urease, and morphology was determined by reaction to Gram’s stain.
Genomic DNA extraction for 16S rRNA gene sequencing. Bacteria isolated from the feces of two mice were cultured on blood agar plates, and the cells were harvested and washed twice with 1 ml of double-distilled H2O. The pellets were suspended in STE buffer (0.9% sucrose, 50 mM Tris-HCl pH 8.0), and lysozyme (hen egg white; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added to a final concentration of 3 mg/ml. The suspension was incubated for 12 min at 37°C and then lysed with 1% sodium dodecyl sulfate. RNase A (bovine pancreas; Boehringer Mannheim) was added to a final concentration of 0.05 mg/ml, and the solution was incubated for 1 h at 37°C. Then 0.1 volume of a 5% (cetyltrimethylammonium bromide–0.5 M NaCl solution (Sigma Chemical Co., St. Louis, Mo.) was added, and the solution was gently mixed and incubated at 65°C for 10 min. DNA was extracted with an equal volume of phenol-chloroform (1:1, vol/vol), precipitated overnight in 0.3 M sodium acetate with 2 volumes of absolute ethanol at −20°C, and pelleted by centrifugation at 13,000 x g for 1 h at 4°C. The ethanol was decanted, and the pellet was air dried and suspended in sterile distilled water.

16S rRNA gene sequencing. Sequences of the 16S rRNA genes of two bacterial isolates (MIT 97-6810 and MIT 97-6811) were determined. For amplification of 16S rRNA cistrons, 16S rRNA gene sequencing, and 16S rRNA data analysis, we used the methods described by Fox et al. (6). Briefly, primers C70 and B37 (6) were used to amplify the 16S rRNA genes. The amplicons were purified and directly sequenced by using a TACQueN cycle sequencing kit (U.S. Biochemical, Cleveland, Ohio). The 16S rRNA gene sequences were entered into a program for analysis of 16S rRNA data in Microsoft Quickbasic for use with PC-compatible computers and were aligned as previously described (16). The database used contains approximately 100 Helicobacter, Wolinella, Arcobacter, and Campylobacter sequences and more than 900 sequences for other bacteria. Similarity matrices were constructed from the aligned sequences by using only sequences in which 100% of the positions for which 90% of the strains had data and were corrected for multiple base changes by the method of Jukes and Cantor (9). Phylogenetic trees were constructed by the neighbor-joining method (18).

Electron microscopy. The novel Helicobacter sp. was examined by electron microscopy. Cells grown on blood agar plates were centrifuged and suspended in 10 mM Tris-HCl buffer (pH 7.4) at a concentration of about 105 cells per ml. Samples were negatively stained with 1% (wt/vol) phosphotungstic acid (pH 6.5) for 20 to 30 s. Specimens were examined with a JEOL model JEM-1200EX transmission electron microscope operating at 100 kV.

Experimental infection with the novel Helicobacter sp. The non-SPF IL-10−/− mice were rederived by cesarean section at Taconic Farms (Germantown, N.Y.). Following rederivation, the mice were backcrossed 7 to 10 times to a C57BL/10 SgNai background. The SPF status of these mice is defined by failure to measure detectable immunoglobulin G (IgG) antibodies to the following murine viruses: mouse hepatitis virus, EDIM virus, MVM, MPV, Sendai virus, PVM, REO-3 GD-VII, lymphocytic choriomeningitis virus, K virus, mouse adenovirus; ectromelia virus, polyomavirus MCMV, and thymus virus. The animals were negative for ecto- and endoparasites. Fecal cultures were negative for parovirus, encephalomyocarditis virus, K virus, mouse ad- enovirus, ectromelia virus, polyomavirus MCMV, and thymus virus. The animals were negative for ecto- and endoparasites. Fecal cultures were negative for Salmonella sp., Campylobacter rodentium, and Klebsiella sp. Fecal PCR was negative for Enteric Campylobacter and H. bilis. Offspring produced were backcrossed 7 to 10 times to a C57BL/10 SgNai background.

Infection of mice. Six male 2-month-old A/JNCr, 6 SCID/NCr, and 11 IL-10−/− mice were inoculated i.p. with 106 bacteria as measured by spectrophotometry (7). The bacteria were suspended in 0.2 ml of phosphate-buffered saline (PBS) by intraperitoneal (i.p.) injection (six mice) and oral gavage (six mice). These mice were euthanized 4 to 8 weeks later (Table 1).

TABLE 1. Incidence ofecal and colon lesions in mice infected with urease-negative Helicobacter sp.*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Route of injection</th>
<th>Time (mo) p.i.</th>
<th>No. with intestinal lesion/no. sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL 10−/−</td>
<td>i.p.</td>
<td>1</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7/7</td>
<td></td>
</tr>
<tr>
<td>Intragastric</td>
<td>i.p.</td>
<td>1</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2/2</td>
<td></td>
</tr>
</tbody>
</table>

* Any mice not noted but injected either died or were used for culture only. None of the control mice hadecal or colon lesions.

Minimal inflammation.

RESULTS

Characterization of the novel Helicobacter species. (i) Bacterial isolation. Urease-negative, catalase- and oxidase-positive, gram-negative bacteria grown at 37°C under microaerobic conditions were isolated from the ceca of the two IL-10−/− mice sampled from the colony with endemic IBD.

(ii) 16S rRNA analysis. The 16S rRNA sequences determined for both urease-negative Helicobacter sp. mouse isolates were entered into our database, aligned, and compared with the over 100 Helicobacter sequences in the database to determine similarity. The sequences of the two urease-negative Helicobacter sp. strains (MIT 97-6810 and MIT 97-6811) were identical to one another and most closely related (97.5% similar) to those of H. muriidenum and H. hepaticus (Fig. 1). The
2.5% difference from other described species indicates that they represent a novel species. Both *Helicobacter* sp. strains contain a 166-base transcribed intervening sequence in place of the 7-base stem-loop that is normally centered at position 210 (*Escherichia coli* numbering).

(iii) **Ultrastructure.** The novel *Helicobacter* sp. was motile and curved to spiral, and it measured 0.3 by 2 to 5 μm (Fig. 2). The bacterium possessed single bipolar sheathed flagella but did not have periplasmic fibers.

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**Experimental infections.** (i) **Identification of Helicobacter sp. by PCR.** A urease-negative, oxidase-positive, catalase-positive, gram-negative spiral-shaped organism was consistently isolated from feces and ceca of experimentally infected mice during the prescribed time points of the experiment and at necropsy.

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**FIG. 1.** Phylogenetic tree constructed on the basis of 16S rRNA sequence similarity values, using the neighbor-joining method. Scale bar = 5% difference in nucleotide sequences as determined by measuring the lengths of the horizontal lines connecting two species.

**FIG. 2.** Phototungstic acid negative stain of urease-negative *Helicobacter* sp. depicting helical bacteria with bipolar sheathed flagella. Magnification, ×13,650.

**FIG. 3.** Agarose gel electrophoresis of DNA amplified by PCR with *Helicobacter* species-specific primers JGF-F1 and JGF-R1 (A) or *Helicobacter* genus-specific primers C97 and C98 (B). Lanes 1 to 8, novel urease-negative *Helicobacter* sp. isolates MIT 97-6810 and MIT 97-6811 and isolates 98-6781, 98-6782, 98-764, 98-7685, 98-7686, and 98-7687, respectively; lane 9, *H. rodentium* 95-1707; lane 10, control with no DNA template; M, 100-bp DNA ladder (GibcoLife Technologies, Gaithersburg, Md.). The 0.6-kb (A) and 0.4-kb (B) positions are indicated by asterisks.

**FIG. 4.** U− HelAg-specific Ab in sera of urease-negative *Helicobacter*-infected mice. Sera from 5.5-month-infected A/J (●) and SCID (▲) mice (A) and from 4.5-month-infected IL-10−/− (■) mice (B) were analyzed for levels of total IgG reactive to U− HelAg by ELISA as described in Materials and Methods. Symbols represent means ± standard deviations of duplicate ELISA values from pools of three infected A/J, two infected SCID, and seven infected IL-10−/− mice, respectively. Uninfected IL-10−/− mice showed no reactivity to the U− HelAg (data from a separate experiment, not shown). OD, optical density.
Helicobacter sp. was not isolated from control mice at any time point during the experiment.

PCR products from the DNA templates were separated on a 1.5% agarose gel (Fig. 3). Primers JGF-F1 and JGF-R1 amplified a 0.6-kb PCR product from all isolates of the novel urease-negative Helicobacter sp. (Fig. 3A, lanes 1 to 8), whereas a similar product was not amplified from H. rodentium 95-1707 (Fig. 3B, lane 9). However, with the universal Helicobacter sp. primers, a 0.4-kb PCR product was amplified from both the newly isolated Helicobacter sp. and H. rodentium (Fig. 3B, lanes 1 to 9).

(ii) ELISA. To analyze humoral immune responses to the urease-negative Helicobacter sp., we bled A/JNCr, SCID/NCr, and IL-10⁻/⁻ mice 4.5 to 5.5 months after inoculation with the urease-negative Helicobacter sp. and analyzed sera for total anti-U-HelAg IgG by ELISA. A/JNCr and IL-10⁻/⁻ mice showed high titers of total anti-U HelAg IgG, whereas, as expected, no helicobacter-reactive Ab was detected in sera from infected SCID mice (Fig. 4). Uninfected IL-10⁻/⁻ mice from a separate experiment also showed no reactivity to the U HelAg (data not shown).

(iii) Histopathology. None of the experimentally infected mice developed diarrhea or rectal prolapse. Although not as severe as the intestinal lesions noted in the IL-10⁻/⁻ mice naturally infected with the urease-negative Helicobacter sp. (Fig. 5), moderate to severe large bowel lesions were noted in all IL-10⁻/⁻ mice inoculated with the novel helicobacter. Route of infection did not influence lesion severity. Uninfected controls had no intestinal lesions (Fig. 6A), whereas each infected mouse had moderate to marked typhlitis, mild colitis, and moderate proctitis. Some mice, examined 6 months p.i., had focal areas of atypical hyperplasia in the cecum (Fig. 6B) and rectum (Fig. 7). Livers of the IL-10⁻/⁻ infected with the novel helicobacter had few to many foci of granulomatous inflammation and mild cholangitis. With Steiner stain, many helical organisms were seen within crypts of the large bowel, especially the cecum. Organisms, however, were not observed in the livers of infected mice.

At 4 weeks p.i., mild to moderate inflammatory and hyperplastic lesions were found in the ceca but not colons of the SCID/NCr mice. Lesions at 6 months p.i. were comparable to those noted at 4 weeks p.i. The liver was normal except for a mild cholangitis. In A/JNCr mice at 4 weeks p.i., no large bowel lesions were found. One mouse had a few liver granulomas. Minimal to mild typhlitis and scattered liver granulomas and foci of cholangitis were noted in the A/JNCr mice examined at 6 months p.i.

DISCUSSION

Although IBD has been characterized in IL-10⁻/⁻ mice, previous reports had implicated normal enteric bacteria as responsible for eliciting the proinflammatory response (10). In this study we isolated and characterized a novel urease-negative helicobacter in IL-10⁻/⁻ mice with IBD. The development of experimentally induced IBD in several different strains of helicobacter-free mice, including IL-10⁻/⁻ mice injected with the urease-negative Helicobacter sp., adds further support to the view that Helicobacter spp. can induce intestinal inflammation in murine hosts. Also, the presence of humoral IgG antibody response to the urease-negative Helicobacter sp. may indicate that the organism was responsible for the intestinal

FIG. 5. Diffuse and focal epithelial hyperplasia with marked inflammation in the cecum of a IL-10⁻/⁻ mouse naturally infected with the urease-negative Helicobacter sp. H&E; magnification, ×30.

FIG. 6. (A) Normal cecum of a 4-month-old uninfected control IL-10⁻/⁻ mouse. H&E stain × 75. (B) Focal atypical hyperplasia and diffuse hyperplasia with marked chronic inflammation in cecum of an IL-10⁻/⁻ mouse, 6 months after infection with the novel urease-negative Helicobacter sp. H&E; magnification, ×30.
the disease. For example, when IL-2
intestinal bacteria appeared to be involved in the pathogenesis of under SPF conditions and absent in the germfree state, intes-
evertheless, as the intestinal inflammation generally was less severe
reported to be free of known murine pathogens (10, 17). How-
mice which are genetically and/or immunologically compro-
mits, housed under SPF conditions (10). At the time of these stud-
by hysterectomy and maintained under SPF or germfree con-
noninfectious).

Similar findings were recorded for IL-10
intestine, but lesions were completely absent from the colonic

Lesions consistent with IBD are increasingly recognized in mice which are genetically and/or immunologically compro-
mice in which the IBD was less severe and delayed in onset when mice were housed under SPF conditions (10). At the time of these stud-
were identified in murine hosts. Since the description of H. hepaticus in 1994, it has been established that H. hepaticus colonizes a large number of mice, from commercial as well as academic sources (19). Indeed H. hepaticus has been isolated from multiple genetically altered mice with IBD (4). Experimental evidence confirming the relevance of H. hepaticus in the induction of IBD was described in a study where H. he-
hepaticus inoculated into defined-flora SCID mice reconstituted
mice also produced severe colitis and typhlitis (21). A more
recent study indicates that helicobacter-free IL-10−/− mice
mice experimentally infected with the novel urease-negative Hel-
cobacter sp. were similar to those associated with H. hepaticus
in naturally and experimentally infected A/JCr and SCID mice
(7). In a subsequent experiment, H. bilis inoculated into def-
defined-flora SCID mice without reconstitution of CD45RB+ T
cells. Another likely possibility is that the IL-

Lesions developed in the large intestines of the IL-10−/−
mice experimentally infected with the novel urease-negative Helicobacter sp., but they were not as severe as those in the
mice infected for shorter time periods (28). H. hepaticus induced a strong proinflammatory Th1 cytokine response and elevated gamma interferon in both IL-10−/− and
mice (11, 28). Interestingly, the novel Helicobacter sp.,
which unlike H. hepaticus and H. bilis lacks urease activity,
elicits a similar Th1 cytokine response in experimentally in-
ected IL-10−/− mice (10a).

Lessons learned from the newly described mouse model of helicobacter-associated intestinal disease.

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