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Helicobacter typhlonius and *Helicobacter rodentium* Differentially Affect the Severity of Colon Inflammation and Inflammation-Associated Neoplasia in IL10-Deficient Mice

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Infection with *Helicobacter* species is endemic in many animal facilities and may alter the penetrance of inflammatory bowel disease (IBD) phenotypes. However, little is known about the relative pathogenicity of *H. typhlonius*, *H. rodentium*, and combined infection in IBD models. We infected adult and neonatal IL10$^{-/-}$ mice with *H. typhlonius*, *H. rodentium*, or both bacteria. The severity of IBD and incidence of inflammation-associated colonic neoplasia were assessed in the presence and absence of anti*Helicobacter* therapy. Infected IL10$^{-/-}$ mice developed IBD with severity of noninfected (minimal to no inflammation) < *H. rodentium* < *H. typhlonius* < mixed *H. rodentium* + *H. typhlonius* (severe inflammation). Inflammation-associated colonic neoplasia was common in infected mice and its incidence correlated with IBD severity. Combined treatment with amoxicillin, clarithromycin, metronidazole, and omeprazole eradicated *Helicobacter* in infected mice and ameliorated established IBD in both infected and noninfected mice. Infection of IL10$^{-/-}$ mice with *H. rodentium*, *H. typhlonius*, or both organisms can trigger development of severe IBD that eventually leads to colonic neoplasia. The high incidence and multiplicity of neoplastic lesions in infected mice make this model well-suited for future research related to the development and chemoprevention of inflammation-associated colon cancer. The similar antiinflammatory effect of antibiotic therapy in *Helicobacter*-infected and -noninfected IL10$^{-/-}$ mice with colitis indicates that unidentified microbiota in addition to *Helicobacter* drive the inflammatory process in this model. This finding suggests a complex role for both *Helicobacter* and other intestinal microbiota in the onset and perpetuation of IBD in these susceptible hosts.

Abbreviation: IBD, inflammatory bowel disease.

Inflammatory bowel disease (IBD) is hypothesized to develop due to aberrant immune responses induced by gut microbes.[5] IBD does not occur in germ-free IL10$^{-/-}$ mice,[2,15] indicating the importance of microorganisms as environmental triggers of intestinal inflammation. However, conventionally colonized or specific pathogen-free IL10$^{-/-}$ mice may develop colitis spontaneously[2,32] or in response to specific triggers such as nonsteroidal antiinflammatory drugs[3,14] or infections with certain bacteria.[6,16,18] The normal lack of ongoing immune responses against bacteria in subjects without IBD has been attributed to the immunologic tolerance that specifically downregulates immune responses against antigens derived from these bacteria. Nevertheless, despite a large number of studies, no single bacterial type has fulfilled Koch postulates and been confirmed as a cause of IBD in animals or humans.

Previous studies used fluorescence in situ hybridization with probes specific for bacterial 16S rRNA combined with conventional histologic techniques to study the relationships between various species of intestinal bacteria and the mucosa in mice and humans with IBD.[33,34] Those studies showed that in normal mice, most bacterial groups are separated from the mucosal surface by either a mucus layer that excludes bacteria or, in the cecum and proximal colon, by an 'interlaced' layer that is composed of tightly packed bacteria. The interlaced or mucus layer thus limits the contact of the bulk of the enteric bacteria with the mucosal epithelium. In contrast, complex biofilms composed of multiple species of bacteria that were firmly adherent to the mucosal surface were identified in the majority of colon tissue samples collected from humans and mice with IBD.[33,34] The presence of a biofilm abrogates the protective effects of the normal layer of mucus and can allow luminal bacterial antigens and toxins to reach the unshielded epithelial surface, where they can trigger cascades of host inflammatory responses. Situations that cause defects in the epithelial surface or degrade the protective qualities of mucus or the interlaced layer (or both) may allow contact of bacterial antigens and adjuvants with immune cells located in the lamina propria and lead to the generation of immune responses that result in IBD.[34]

Helicobacter species are used frequently to model microbial triggers of colon inflammation, because they have previously been linked to the development of both IBD- and inflammation-associated neoplasia.[11,21,29] Most studies have been performed by using *Helicobacter hepaticus* or *H. bilis*.[20] However, *H. typhlonius*,

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H. rodentium, *H. muridarum*, *H. ganmani*, *H. trogontum* and other species[8,12,17,29,35] can also be endemic in research animal facilities. The pathophysiologic effects of these less-common *Helicobacter* species are, for the most part, poorly investigated.

Most rodent *Helicobacter* species are urease-negative and therefore preferentially colonize the intestine, but some species produce urease enzyme and can translocate to the liver or colonize the biliary system.[13] *H. typhlonius* was shown to cause an enteric disease characterized by mucosal hyperplasia and associated inflammation in the cecum and colon in immunodeficient mice[11,23] and IL10$^{-/-}$ mice.[18] *H. typhlonius* is genetically related most closely to *H. hepaticus*, having only 2.36% difference in the 16S rRNA gene sequence, but *H. typhlonius* has a unique intervening sequence in this gene that makes it easily recognizable by PCR.[9,12] Molecular detection of this pathogen with PCR is rapid, sensitive and allows the detection of the early phases of infection; further enhanced sensitivity is achieved with nested primers.[22] One of the most important features of PCR is that it can be performed noninvasively on fecal pellets. Data regarding the pathogenetic mechanisms of *H. rodentium* are scarce.[35,36] *H. rodentium* alone apparently does not cause hepatitis or enteritis in A/JCr or C.B-17/IcrCrl-scidBr mice; however, coinfection with *H. hepaticus* and *H. rodentium* was associated with augmented cecal gene expression and clinical diarrheal disease in immunodeficient mice compared with mice infected with *H. hepaticus* alone.[23]

Previous reports demonstrated that *H. typhlonius* was capable of initiating colitis in adult IL10$^{-/-}$ mice.[10,11] In those studies, colitis was relatively mild, with no development of inflammation-associated neoplasia. *H. rodentium* has been described to be nonpathogenic in adult wild-type mice but did enhance cytokine production in the cecum of mice also infected with *H. hepaticus*.[23] We recently observed rapid onset of severe IBD and a high incidence of inflammation-associated neoplasia in IL10$^{-/-}$ mice that were coinfected with both *H. typhlonius* and *H. rodentium* as pups.[16] The current study was undertaken to determine the relative roles of *H. rodentium* and *H. typhlonius*, individually and in combination, and age at infection in the development of colon inflammation and inflammation-associated neoplasia in IL10$^{-/-}$ mice. Novel features of our model include controlled infection of the combination of *H. typhlonius* and *H. rodentium*[9] and infection of IL10$^{-/-}$ mice during the neonatal period.

Materials and Methods

Animals and husbandry. Specific pathogen-free IL10$^{-/-}$ male and female mice on the C57BL/6 background (strain name = B6.129P2-Il10^{tm1Cgn}/J; stock # 002251) were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were confirmed to be *Helicobacter*-free by PCR using a genus-specific primer. Mice were housed in polycarbonate microisolation caging in ventilated isolation units or on individually ventilated racks under BSL2 conditions, with access to food and water ad libitum. Mice were observed daily for clinical signs of distress, and weight was monitored 3 times per week. Humane endpoints included loss of more than 15% of body weight or development of rectal prolapse, a well-recognized complication of chronic inflammation in the colon. The success of this study was dependent on stringent husbandry techniques to prevent cross-contamination. These techniques included a strictly enforced order of cage handling and scrupulous attention to environmental sanitization. All mice were consistently negative for all except the intentionally introduced *Helicobacter* spp. All animal studies were approved by the Duke University Institutional Animal Care and Use Committee.

***Helicobacter* infection.** Mice were infected on day 0 with *H. typhlonius* (clinical isolate DU-01),[16] *H. rodentium* (MIT 95-1707; equal to ATCC type strain 700285)[29] by gavage of a single dose of 500 µl culture (approximately 5×10^7 organisms unless otherwise specified). For inoculation, both strains were grown in *Brucella* broth (Becton Dickinson, Franklin Lakes, NJ) as previously described.[19] Cultures were agitated with a stir bar in a 250-ml Erlenmeyer flask and were incubated for 24 h at 37 °C in a an atmosphere of 90% N_2, 5% H_2, and 5% CO_2. Noninfected controls were sham-gavaged or received sterile broth or PBS only. Because initial experiments showed frequent failure of sustained *H. rodentium* infection when *H. rodentium* and *H. typhlonius* were given simultaneously, an additional dose of *H. rodentium* was given on day 2 to mice in the mixed-infection group. Infection was confirmed at 1 wk after infection and at 4-wk intervals thereafter by analysis of feces by PCR (see *PCR of* Helicobacter *organisms*). Breeding to generate neonatally infected mice typically began immediately after infection and was performed in triads consisting of 1 male and 2 female mice. Infection in pups born to infected dams was confirmed 1 to 2 wk postweaning. Mice were euthanized by CO_2 asphyxiation[1] if they developed 15% body weight loss or rectal prolapse or when they reached 7 to 8 months of age. All mice in this study were evaluated pathologically for both colitis and neoplasia. Sentinel mice exposed repeatedly to dirty bedding from the mice used in this study were negative for parasites by microscopic exam, negative for *Citrobacter rodentium* by fecal culture, and negative by serology for a panel of 22 murine protozoal, bacterial, and viral pathogens, including murine parvovirus, murine hepatitis virus, and murine norovirus. The studies were performed at an AAALAC-accredited institution. All studies were performed in accordance with *The Guide for the Care and Use of Laboratory Animals*.[24]

Anti*Helicobacter* therapy. Mice designated to receive an anti-*Helicobacter* therapy treatment received commercially available wafers containing 3 mg amoxicillin, 0.5 mg clarithromycin, 1 mg metronidazole, and 20 µg omeprazole per 5 g of food (BioServ, Frenchtown, NJ). Mice were maintained on anti*Helicobacter* wafers until euthanasia for tissue harvest.

To analyze the effects of anti*Helicobacter* therapy on noninfected IL10$^{-/-}$ mice with colitis, the mice were given 200 ppm piroxicam for 7 d to trigger development of chronic colitis and then were treated with the 4-drug anti*Helicobacter* therapy combination for 16 d.

Sample collection. After euthanasia, the digestive tract from stomach to anus was removed and divided into segments representing the stomach, proximal, mid-, and distal small intestine, cecum, and proximal, mid-, distal, and terminal colon and rectum. Other organs collected to assess the extent of infection were mesenteric lymph nodes, spleen, liver, pancreas, stomach, ovary, and lung. Portions of each gastrointestinal segment were rinsed briefly with PBS to remove nonadherent organisms. Tissues for molecular analysis were frozen immediately and stored at –20 °C for subsequent quantitation of associated *Helicobacter* organisms by quantitative real-time PCR. The remaining tissues were fixed in Carnoy solution for 2 to 4 h and then processed and embedded into paraffin.

PCR detection of *Helicobacter* organisms. DNA was extracted from weighed fecal and tissue samples by using the DNeasy Tis-

sue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, samples were thawed in 180 µl ATL buffer and 20 µl proteinase K were added and then incubated overnight at 56 °C. Next, 200 µl AL buffer was added to the tube, the sample was vortexed, and 200 µl 100% ethanol was added. Finally, sample was loaded onto the silica-gel column, washed, and eluted in 100 µl elution buffer. To quantify the relative concentrations of fecal and mucosa-associated *H. rodentium* and *H. typhlonius* organisms, DNA samples were probed with primers designed to amplify segments of the *H. rodentium* Ni/Fe hydrogenase or *H. typhlonius cdtB* genes.[28] A standard curve for quantification of the 2 *Helicobacter* strains was generated from serial dilutions of bacterial DNA and used to calculate numbers of bacterial copies per gram of tissue or feces.

Estimates of the number of *Helicobacter* genome copies in the standard were based on a genome size of 1.8 megabases and a molecular mass of 1.09×10^9 Da. The PCR reactions and melting curves were performed in 20 µl that contained 0.5 µl of each primer, 10 µl SYBR Green PCR Master Mix (Stratagene, La Jolla, CA), and 4 µl sample DNA. The PCR reaction was incubated at 95 °C for 15 min to activate the polymerase followed by 40 cycles consisting of denaturation for 15 s at 94 °C, annealing for 20 s 58 °C, and extension for 30 s at 72 °C. Fluorescence was monitored at the end of each extension phase. After amplification, melting curves were generated to verify PCR product identity.

Histologic scoring. The severity of colonic inflammation and incidence of colon neoplasia seen in hematoxylin and eosin-stained sections was scored by a pathologist blinded to treatment group. Histologic scores were calculated (as described in reference 15 and modified from reference 6) by using a scale that takes into account mucosal changes in 5 different bowel segments, including hyperplasia and ulceration, degree of inflammation, and percentage of each bowel segment affected by these changes. With this scale, the maximum score is 75, and a score greater than 12 indicate the presence of colitis. Sections also were scored for neoplasia according to a consensus report and recommendations.[4] Gastrointestinal intraepithelial neoplasia (synonymous with atypical hyperplasia, microadenoma, and carcinoma in situ) and adenoma were considered to be noninvasive lesions. Invasive lesions were classified as adenocarcinoma.

Statistical analysis. Statistical comparison of groups was performed by using Student *t* tests or ANOVA. Survival rates were calculated by using the Kaplan–Meier test with *P* values calculated by using the log-rank test. A *P* value of less than or equal to 0.05 was considered to be significant.

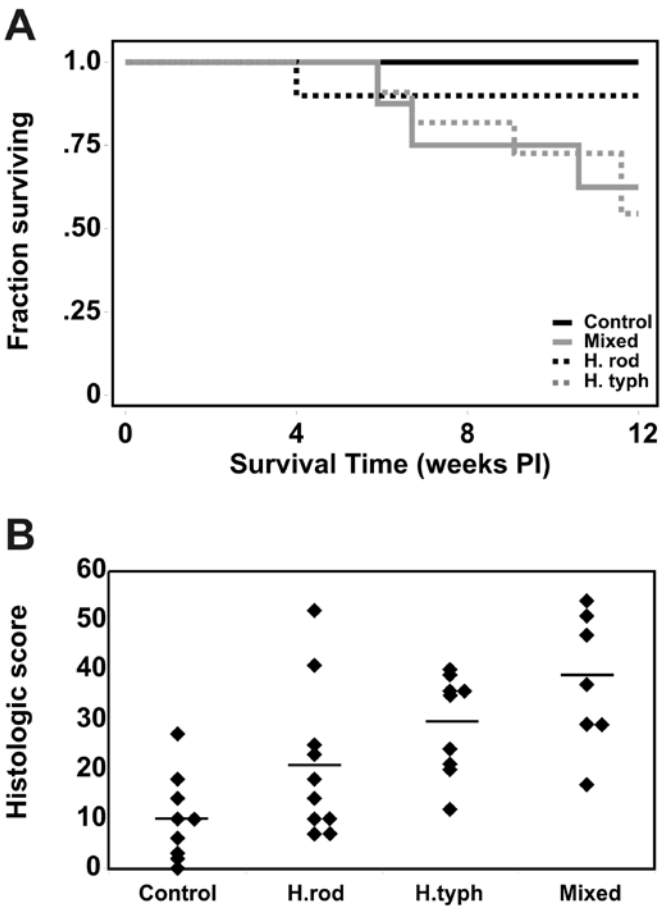

Figure 1. Short-term survival and colitis severity in *Helicobacter*-infected adult IL10$^{-/-}$ mice. (A) Kaplan–Meier plot shows survival rate of adult IL10$^{-/-}$ mice infected with *H. rodentium* (H. rod), *H. typhlonius* (H. typh), or both (mixed) bacteria (short-term study). Control, *H. rodentium*-, *H. typhlonius*-, and mixed *H. rodentium* + *H. typhlonius*-infected groups contained 10, 10, 11, and 8 mice, respectively. Survival rates in *H. typhlonius*- and mixed infection groups were significantly decreased compared with the noninfected mice ($P = 0.01$ and 0.04, respectively). PI, postinfection. (B) Histologic scores (mean ± SEM) of mice infected at 7 to 8 wk of age and euthanized 11 wk after infection. Control, *H. rodentium*-, *H. typhlonius*-, and combined *H. rodentium* + *H. typhlonius*-infected groups contained 9, 10, 9, and 7 mice, respectively. Mice infected with *H. typhlonius* or both *H. rodentium* and *H. typhlonius* had significantly higher histologic scores than did the noninfected control group ($P = 0.0005$ and 0.0009, respectively, by Student *t* test).

Results

Infection with *H. typhlonius* with or without *H. rodentium* markedly increases the severity of IBD in adult IL10$^{-/-}$ mice. IL10$^{-/-}$ mice at 6 to 8 wk of age were infected with either *H. typhlonius*, *H. rodentium*, or both and then observed for as long as 82 d before histologic determination of IBD severity. Histologic scores showed marked differences in colonic inflammation between various infection groups (Figure 1 B). Control IL10$^{-/-}$ mice did not develop inflammation during this time period (mean histologic score ± SEM, 10 ± 3; n = 9). In contrast, mice infected with *H. rodentium* or *H. typhlonius* or both developed colonic inflammation. Mice infected with *H. rodentium* alone developed mild to moderate colitis (21 ± 5; n = 10). Mice infected only with *H. typhlonius* had histologic scores of 29 ± 3 (n = 9; $P = 0.0005$ versus noninfected controls), whereas those infected with both *H. rodentium* and *H. typhlonius* had histologic scores of 38 ± 5 (n = 7; $P = 0.0009$ versus noninfected controls). Inflammatory changes were noted in all portions of the colon in these mice but were most severe in the cecum, followed by the proximal colon and terminal colon and rectum.

Some infected mice developed rectal prolapse during the course of this study and required euthanasia for humane reasons. The mean survival rate for the mice infected with *H. typhlonius* was significantly different than that of noninfected controls ($P = 0.017$; Figure 1 A), whereas the mean survival of mice infected with *H. rodentium* was not statistically different ($P = 0.3$) from that of noninfected controls. Overall, the severity of colitis in these *Helicobacter*-infected mice was higher than that seen in other

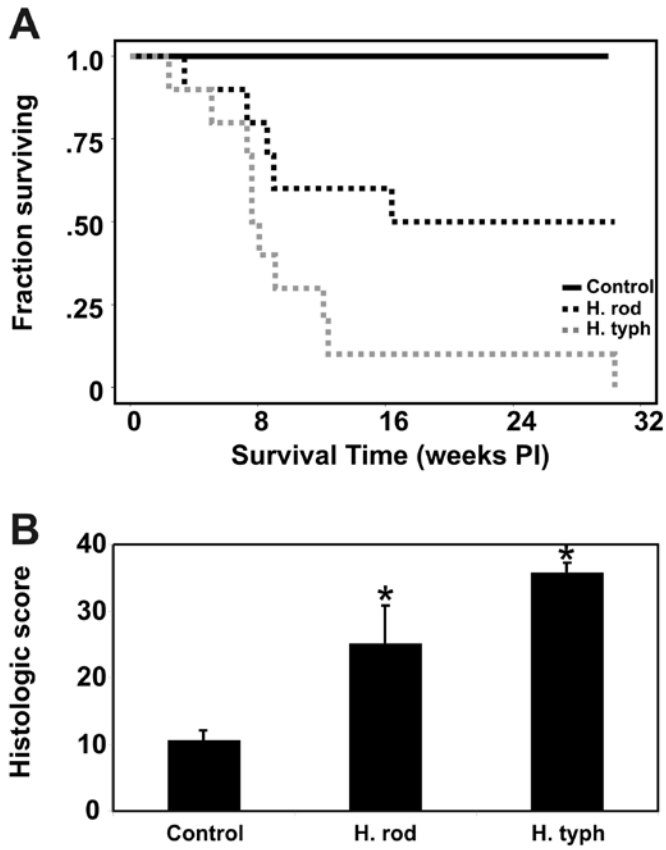

Figure 2. Long-term survival and colitis severity in *Helicobacter*-infected adult IL10$^{-/-}$ mice. (A) Kaplan–Meier plot shows survival rate of adult IL10$^{-/-}$ mice (n = 10 for each group) infected with *H. rodentium* (H. rod) or *H. typhlonius* (H. typh) in long-term experiments. Survival was significantly decreased in mice infected with either *H. rodentium* or *H. typhlonius* compared with noninfected control mice ($P < 0.01$ and 0.0001, respectively). (B) Histologic scores (mean ± SEM) of mice (n = 10 for each group) infected at 7 to 8 wk of age. Mice infected with *H. rodentium* or *H. typhlonius* had significantly higher histologic scores compared with the control group ($P = 0.02$ and < 0.001, respectively, by Student *t* test).

murine models of IBD, including piroxicam-triggered colitis in IL10$^{-/-}$ mice.[14] Although the mean histologic score in mice infected with *H. rodentium* was not statistically different from that seen in control mice in light of the wide variability of inflammation severity in the *H. rodentium* group, some mice in that group had severe IBD. Therefore, *H. rodentium* is capable of triggering IBD in IL10$^{-/-}$ mice. Colonic neoplastic lesions were not detected during this short-term experiment.

To further address the contribution of these specific *Helicobacter* species to colitis severity and inflammation-associated colonic neoplasia, additional cohorts of adult IL10$^{-/-}$ mice were infected with *H. rodentium* or *H. typhlonius* and monitored for as long as 7 mo postinfection. All control noninfected mice survived to 28 wk of age and demonstrated minimal to no intestinal inflammation at this time point (mean histologic score ± SEM, 11 ± 1; n = 10; Figure 2 B). In contrast, 50% of mice infected with *H. rodentium* and 100% of mice infected with *H. typhlonius* developed rectal prolapse that required euthanasia for humane reasons prior to the scheduled end point at 28 wk of age (Figure 2A). Mean survival for mice infected with *H. rodentium* was 17 ± 3 wk ($P = 0.01$ versus noninfected), with corresponding mean survival of 10 ± 2 wk for mice infected with *H. typhlonius* ($P = 0.0001$ versus noninfected).

The incidence of colonic neoplasia in mice infected with *H. typhlonius* was 50% whereas 40% of the total mice studied had invasive adenocarcinoma (Figure 3 K and L; Figure 4). The incidence of neoplasia tended to increase with time after infection. Therefore the high incidence of rectal prolapse that necessitated early euthanasia likely decreased the frequency of neoplasia. In addition, the incidence of colonic neoplasia decreased as the severity of IBD decreased, with 30% incidence of gastrointestinal intraepithelial neoplasia in mice infected with *H. rodentium* alone. None of these *H. rodentium*-infected mice developed invasive adenocarcinoma.

Effects of infection of pups with *H. typhlonius*, *H. rodentium*, or both species on IBD severity in IL10$^{-/-}$ mice. A previously published study showed an extremely high incidence (95%) of colonic neoplasia at a mean age of 21 ± 2 wk in IL10$^{-/-}$ mice infected with a combination of *H. typhlonius* and *H. rodentium* via exposure to their infected dams.[16] In the current set of experiments, female mice were infected with *H. rodentium*, *H. typhlonius*, or both prior to initiation of pregnancy, and pups were naturally infected by exposure to maternally excreted organisms. Interestingly, mice infected as pups had a decreased incidence of rectal prolapse that necessitated euthanasia compared with mice infected with the same *Helicobacter* species as adults ($P = 0.007$ for *H. rodentium* and *H. typhlonius* infections, compare Figures 2 A and 5 A). Mice infected with these *Helicobacter* species as pups and monitored for as long as 7 mo showed a similar pattern of survival and histologic scores compared with adults. Mice infected with *H. typhlonius* as pups showed significantly lower length of survival (19 ± 1 wk; n = 19) than that of controls ($P < 0.01$; Figure 5A) and higher inflammation scores (mean ± SEM, 42 ± 3; n = 17; Figure 5 B) than did the other groups. The mean length of survival for mice infected with *H. rodentium* as pups was 29 ± 1 wk of age (n = 18; $P = 0.05$ compared with noninfected), allowing development of inflammation-associated neoplasia. In mice infected with *H. typhlonius* as pups, neoplastic lesions were present in 24% of mice examined, with invasive adenocarcinoma present in 18% (Figure 4). Neoplasia was present in 29% of mice infected with *H. rodentium* as pups, with 12% having invasive adenocarcinoma.

Antibiotics eradicated *Helicobacter* infection and decreased severity of IBD in *Helicobacter*-infected mice. To address the role of *Helicobacter* organisms themselves in driving intestinal inflammation, we treated IL10$^{-/-}$ mice infected with *H. typhlonius*, *H. rodentium*, or both as adults with anti*Helicobacter* therapy beginning on day 30 after infection. Mice were euthanized for histologic determination of colon inflammation severity after either 7 or 10 wk of treatment. Fecal PCR showed eradication of detectable fecal excretion of *Helicobacter* DNA by day 7 of anti*Helicobacter* therapy, with fecal PCR remaining negative throughout the remainder of the study. At the time of euthanasia, mice infected with *Helicobacter* but not treated with antibiotics had moderate to severe colitis (Figure 6). After 7 to 10 wk of combination anti*Helicobacter* therapy, the mice previously infected with *Helicobacter* spp. had mild or no colitis, with histologic scores that were statistically similar to antibiotic-treated but noninfected control IL10$^{-/-}$ mice (Figure 6). These histologic scores did not differ from those of noninfected IL10$^{-/-}$ mice without colitis. Euthanasia due to rectal prolapse typically would be necessary for a number of mice in-

fected with these *Helicobacter* species but not treated with antibiotics (compare Figures 1 A and 5 A). However, no rectal prolapse or other cause of mortality occurred in infected, antibiotic-treated mice in this experiment. Therefore, treatment with anti*Helicobacter* therapy decreased the severity of colitis in the *Helicobacter*-infected mice and increased the survival rate in all infected experimental groups.

Anti*Helicobacter* therapy decreased IBD severity in noninfected IL10$^{-/-}$ mice. The combination of antibiotics used to treat mice in the previous experiment was designed to eradicate *Helicobacter* spp. but likely also has effects on other non*Helicobacter* intestinal microbiota that may affect the severity of intestinal inflammation. To address this question, IL10$^{-/-}$ mice confirmed by PCR to be noninfected with *Helicobacter* but with similarly severe IBD triggered by a 7-d exposure to 200 ppm piroxicam in food[3,14] were treated for 16 d with the same 4 drug anti*Helicobacter* therapy combination used in the *Helicobacter*-infected mice. *Helicobacter*-noninfected IL10$^{-/-}$ mice with IBD triggered by piroxicam exposure showed minimal to no colitis after treatment with the anti*Helicobacter* therapy combination for 16 d (mean histologic score ± SEM, 11 ± 3; n = 10; Figure 7). In contrast, noninfected IL10$^{-/-}$ mice with IBD triggered by piroxicam that were not treated with anti*Helicobacter* drugs had moderate to severe colitis at this time point (mean score ± SEM, 31 ± 5; n = 8; $P = 0.006$).

***Helicobacter* infection status of organs in infected mice.** An abundance of data is available regarding infection with *H. bilis* and *H. hepaticus* in the colon of IL10$^{-/-}$ mice,[10,25] but little is known about other *Helicobacter* species including *H. typhlonius* and *H. rodentium*. In addition, most previous studies relied primarily on qualitative analysis of *Helicobacter* presence in feces. However, the anatomic location of infection and the bacterial burden may affect the severity of inflammation and can only be evaluated by direct analysis of tissue. Therefore we analyzed freshly passed feces, contents of the gastrointestinal tract, and a broad range of tissues harvested from *Helicobacter*-infected and control noninfected mice on day 82 postinfection for *Helicobacter* DNA by quantitative real-time PCR (n = 3 mice/group). As expected, both *H. rodentium* and *H. typhlonius* were detected in feces from infected mice (Figure 8). More importantly, these organisms frequently were detected in washed tissue samples from the stomach and all portions of the colon. *H. rodentium* DNA also was detected in multiple samples from the small intestine and in 1 mesenteric lymph node sample. Analysis of the contents of various anatomic regions of the gastrointestinal tract mimicked the distribution of the *Helicobacter* in the tissue (data not shown), with more bacteria in the contents compared with the tissue. Detection of *Helicobacter* DNA in washed tissue samples suggests that some *Helicobacter* organisms adhere strongly to the mucosal surface, whereas detection in feces indicates that others readily pass into the fecal stream.

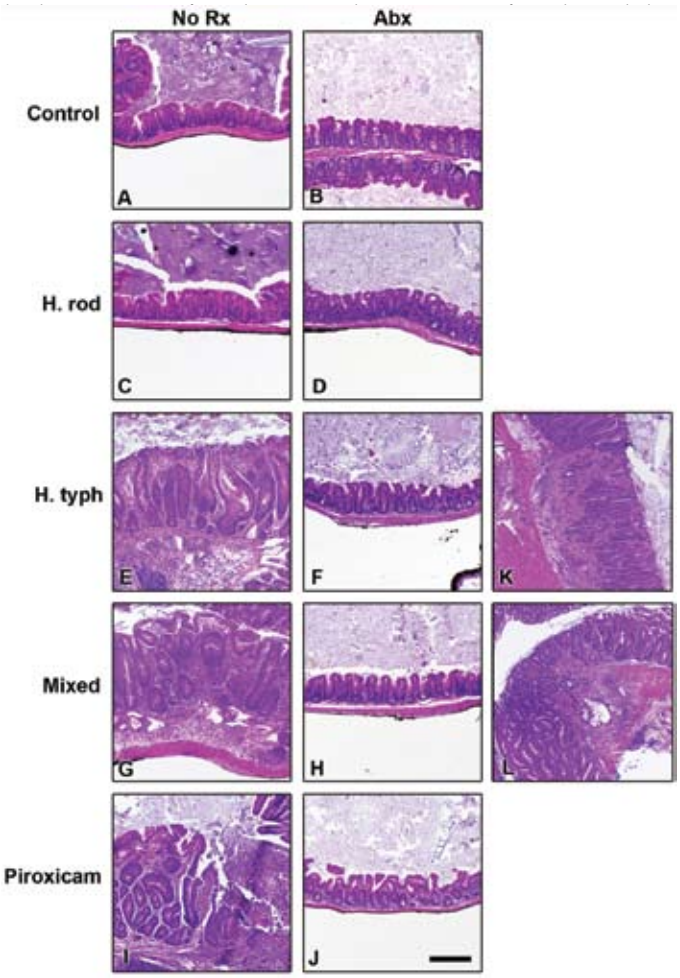

Figure 3. Colon histology and neoplasia in *Helicobacter*-infected and noninfected IL10$^{-/-}$ adult mice. The histologic appearance of the cecum is shown for noninfected control mice (A, B), mice infected with *H. rodentium* (H. rod; C, D), *H. typhlonius* (H. typh; E, F, K), or both (Mixed; G, H, L) and noninfected mice with inflammatory bowel disease triggered by piroxicam (I, J) in the presence (Abx) and absence (no Rx) of treatment with a 4-drug anti*Helicobacter* regimen. Untreated IL10$^{-/-}$ mice infected with *H. typhlonius* or mixed *H. rodentium* plus *H. typhlonius* or noninfected mice exposed to piroxicam had marked mucosal hyperplasia with prominent inflammatory infiltrates that were totally abrogated by anti*Helicobacter* treatment. Inflammatory changes in mice infected with *H. rodentium* alone are not evident at this magnification. Examples of neoplastic lesions seen in long-term *H. typhlonius*-infected (K, proximal colon) IL10$^{-/-}$ mice and mice infected long-term with both *H. typhlonius* and *H. rodentium* (L, cecum) are shown. All photomicrographs were taken at the same magnification; bar, 200 µm.

Discussion

Our results showed increased colon inflammation in IL10$^{-/-}$ mice infected with *H. typhlonius* with or without *H. rodentium*, compared with noninfected mice. Infection with *H. rodentium* alone was also capable of triggering IBD in IL10$^{-/-}$ mice. Compared with that in noninfected mice, survival was decreased in adults infected with *H. typhlonius*, either alone or together with *H. rodentium*. Mice infected with these *Helicobacter* species as pups had qualitatively similar severity of inflammation compared with those infected as adults, however long-term survival was much

Our studies show that the clinical severity of disease is affected by whether *Helicobacter* infection is acquired before (as pups) or after establishing normal endogenous microbiota. In our study, mice infected as pups developed inflammation which resembled that of adults inoculated with the live cultures, but the survival rate of mice infected as pups was much higher and the incidence of inflammation-associated neoplasia was decreased relative to outcomes in the adult study. More studies are necessary to determine the explanations for these observations. Direct infection of adult mice with known concentrations of bacteria may change

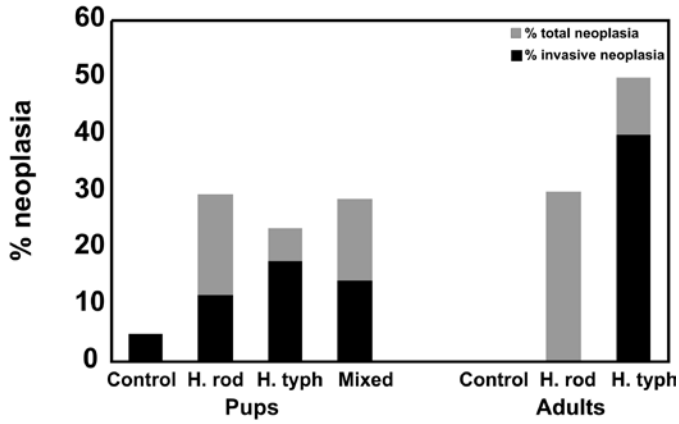

Figure 4. Incidence of neoplasia in IL10$^{-/-}$ mice born to mothers infected with *H. rodentium* (H. rod), *H. typhlonius* (H. typh), or both (Mixed). Gray bars represent mice with only noninvasive gastrointestinal intraepithelial neoplasia (equivalent to dysplasia and carcinoma in situ), whereas black bars represent mice with at least 1 focus of invasive colonic adenocarcinoma. In pups born to *H. typhlonius*-infected mothers, neoplastic lesions were present in 24% mice examined, with invasive adenocarcinoma present in 18%. Neoplasia was present in 29% of pups born to mothers infected with *H. rodentium*, with 12% having invasive adenocarcinoma. Pups with *H. typhlonius* and mixed infection were significantly younger when examined than were control pups (P = 0.008 and 0.001, respectively). In adults, the incidence of colonic neoplasia in mice infected with *H. typhlonius* was 50% and 40% for total neoplasia and invasive adenocarcinoma, respectively. The incidence of colonic neoplasia in adults decreased as the severity of IBD decreased (compare with Figures 1 and 2), with 30% incidence of neoplasia in adult mice infected with *H. rodentium* alone. Invasive adenocarcinoma was not present in control (noninfected) or *H. rodentium*-infected adult mice.

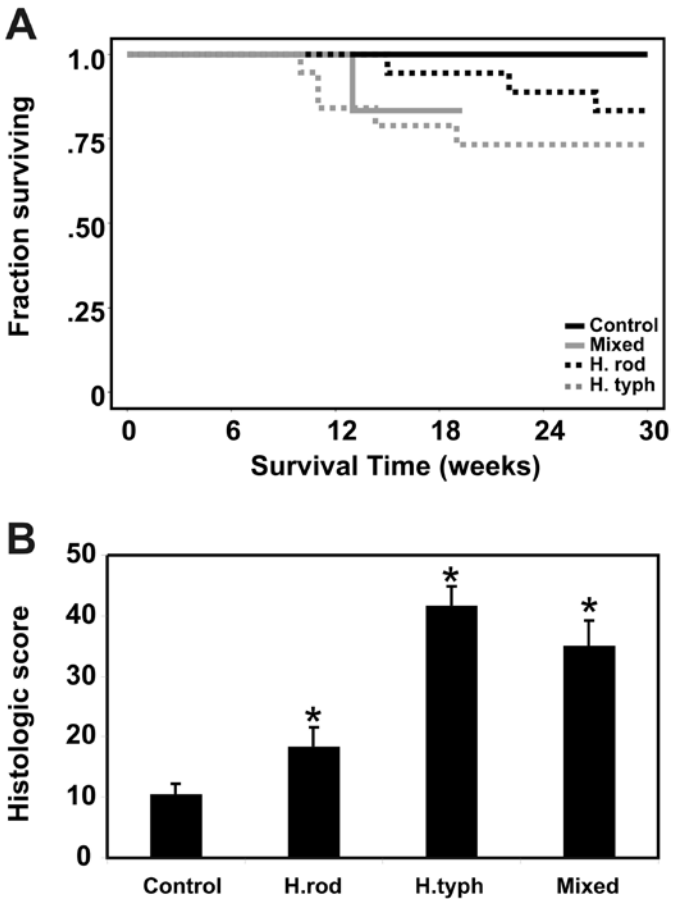

Figure 5. Survival and colitis severity in IL10$^{-/-}$ mice infected as pups. (A) Kaplan–Meier plot showing survival rate of pups born to the mothers infected with *H. rodentium* (H. rod), *H. typhlonius* (H. typh), or both (Mixed). Control, *H. rodentium*-, *H. typhlonius*-, and combined *H. rodentium*- and *H. typhlonius*-infected groups contained 21, 18, 19 and 7 mice, respectively. Pups born to mothers infected with *H. typhlonius* had significantly (P < 0.01) lower survival rate at 18 wk compared with control pups. (B) Histologic scores (mean ± SEM) of pups born to infected mothers. Control, *H. rodentium*-, *H. typhlonius*-, and combined *H. rodentium* and *H. typhlonius*-infected groups contained 20, 17, 17 and 7 mice, respectively. Pups born to dams infected with *H .rodentium*, *H. typhlonius*, or both *H. rodentium* and *H. typhlonius* had significantly higher histologic scores compared with the control group (P = 0.04, 0.001, and 0.01, respectively, by Student t test).

the colonic microenvironment in ways that affected the development of inflammation in that study group. It is possible that mice infected as pups could develop partial resistance to *Helicobacter* pathogenicity before being exposed to normal environmental microbiota; if so, it seems that such resistance did not eliminate the risk of IBD.

This experiment shows that *H. typhlonius* and *H. rodentium* can trigger the development of IBD in IL10$^{-/-}$ mice, although the mechanism remains somewhat unclear. Because bacterial antigens are thought to drive IBD, *H. rodentium* and *H. typhlonius* may burrow through the mucus to grow adjacent to the intestinal epithelial surface, where they degrade the barrier properties, similar to the documented action of *H. pylori* in the stomach.[29] The injurious leakage in this case would be of bacterial antigens and adjuvants that incite immune response that damage the intestine rather than acid (as in mechanism of *H. pylori*), thereby leading to the development of IBD in susceptible host. That anti*Helicobacter* treatment also completely abrogated established intestinal inflammation in the noninfected, piroxicam-treated IL10$^{-/-}$ mice suggests that this anti*Helicobacter* treatment also acts on additional (non*Helicobacter*) bacteria that drive the disease in noninfected mice.

Overall, mice directly infected with *H. typhlonius* as adults had a higher incidence of neoplasia compared with the mice infected as pups through contact with the infected mother (Figure 4), whereas mice infected as adults with *H. rodentium* did not develop invasive carcinoma at all. Interestingly, our previous studies[16] showed a very high incidence of inflammation-associated colonic neoplasia in IL10$^{-/-}$ mice coinfected with *H. typhlonius* and *H. rodentium* as pups (mean of 4 neoplastic lesions per colon in 14 mice examined, with invasive adenocarcinoma present in 57%). The lower number of neoplastic lesions in mice infected with *H. rodentium* as adults in the present study may suggest lower pathogenicity of *H. rodentium* as reported previously.[23] In addition, we show a high rate of neoplasia in mice infected with *H. typhlonius* alone as pups, but the incidence and multiplicity of neoplastic lesions was less than reported previously for mixed infections with both *H. typhlonius* and *H. rodentium*.[16] The combination of *H. rodentium* and *H. typhlonius* may be critical for the early onset and severity of inflammation observed in previous studies.[16]

Helicobacter species have been shown to infect organs of the gastrointestinal tract, including stomach, intestine, and liver.[26,31]

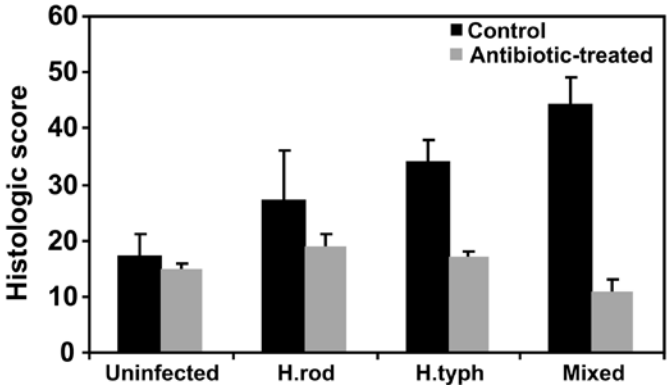

Figure 6. Effect of anti*Helicobacter* therapy on *Helicobacter*-infected IL10$^{-/-}$ mice. Histologic scores (mean ± SEM) are shown for IL10$^{-/-}$ mice infected with *H. rodentium* or *H.typhlonius* at the age of 6 to 8 wk and treated with the 4-drug anti*Helicobacter* combination after day 30 of infection. Treated control, *H. rodentium*-, *H. typhlonius*-, and combined *H. rodentium* + *H. typhlonius*-infected groups contained 7, 9, 9 and 8 mice, respectively. Nontreated control, *H. rodentium*-, *H. typhlonius*-, and combined *H. rodentium* + *H. typhlonius*-infected groups contained 9, 10, 9, and 7 mice, respectively. Survival rates did not differ between the groups of antibiotic-treated mice. Mice were euthanized at 11 wk after infection.

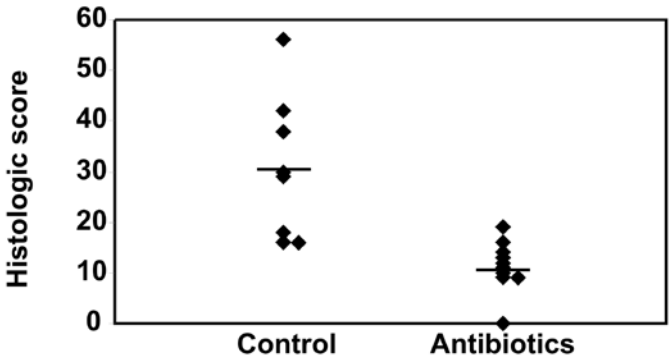

Figure 7. Effect of anti*Helicobacter* therapy on noninfected IL10$^{-/-}$ mice with colitis. Inflammatory bowel disease was induced by exposure to 200 ppm piroxicam for 7 d, after which mice were treated with the 4-drug anti*Helicobacter* therapy combination for 16 d. Mice treated with anti*Helicobacter* therapy had significantly lower histologic scores (mean score ± SEM, 11 ± 3; n = 10) compared with mice not treated with anti*Helicobacter* drugs (31 ± 5; n = 8; $P = 0.006$).

The murine *Helicobacter* species *H. typhlonius* and *H. rodentium* have generally been considered to colonize the cecum. However, the PCR studies reported here demonstrate that the range of infected sites is much broader than previously thought.[16,33] In the present study, we detected both *H. rodentium* and *H. typhlonius* (especially in the mice initially infected with both strains) in the tissue collected from several organs, including stomach. The detection of *Helicobacter* DNA in gastric contents may simply reflect coprophagia. However, detection of *Helicobacter* DNA in washed gastric tissue suggests that the organisms are tightly associated with the mucosa. Furthermore, the levels of *Helicobacter* organisms detected in gastric tissue (5.5×10^7 and 1.7×10^6 organisms per g of tissue for *H. rodentium* and *H. typhlonius*, respectively) seem rather higher than might be expected to be due to coprophagia alone. Although *H. rodentium* and *H. typhlonius* are urease-negative, they

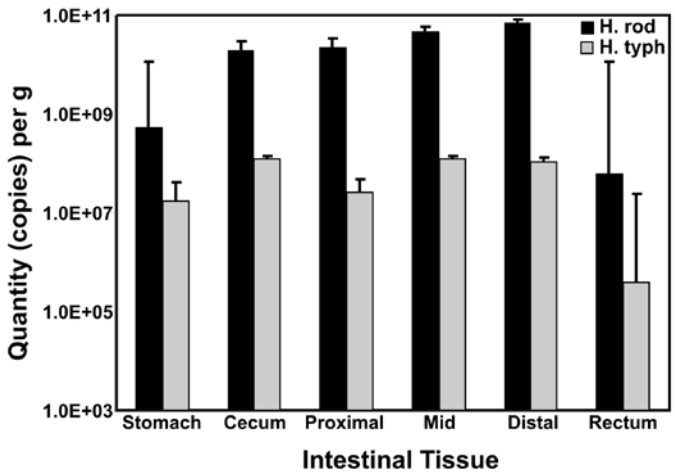

Figure 8. Quantification of *H. rodentium* and *H. typhlonius* in intestinal tissue and stomach. Three IL10$^{-/-}$ mice inoculated with both bacterial strains were studied. Tissue was collected on day 82 of infection. Quantification of the bacterial genomes was based on the presence of Ni/Fe hydrogenase and *cdtB* gene for *H. rodentium* and *H. typhlonius*, respectively. The number of copies of the bacterial genome is expressed per gram of tissue.

have been previously been reported to be associated with gastric mucosa.[7,27] Other investigators[27] detected *H. typhlonius* in the feces, gastric, and intestinal tissues and reproductive organs of wild-type C57BL/6 mice at wk 8 to 10 postinfection. However, the mice in that study were infected naturally, and the inoculum size is unknown. *H. rodentium* and *H. typhlonius* may synergize, thus allowing competitive exclusion necessary for survival in variety of tissues. Note that the PCR assays used in our studies detect bacterial DNA and cannot address the viability of the bacteria detected.

In conclusion, infection with *H. rodentium*, *H. typhlonius*, or both species markedly increased the development and severity of colon inflammation and inflammation-associated colonic neoplasia in IL10$^{-/-}$ mice. The rapid development of IBD makes this model very suitable for the studies on the mechanisms of IBD pathogenesis and treatment. In addition, mice coinfected with either or both organisms as pups may be useful for studies of chemoprevention of IBD-associated colonic neoplasia. IL10-deficient mice produce all lineages of immune cells and mount very strong responses to immune challenges because of their lack of the immunoregulatory cytokine IL10. The demonstration that *H. rodentium* can be pathogenic in these mice that are not classically immunodeficient clearly indicates that lab animal veterinarians should consider this organism to be a pathogen rather than a commensal organism. Because the reliability of an experiment that uses an in vivo model system depends on understanding and controlling all variables that can influence the experimental outcome, unintentional or nonrecognized infections with *H. rodentium* or *H. typhlonius* might interfere with ongoing research studies.

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