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Inflammatory Bowel Disease: an Immunity-Mediated Condition Triggered by Bacterial Infection with *Helicobacter hepaticus*

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Inflammatory bowel disease (IBD) is thought to result from either an abnormal immunological response to enteric flora or a normal immunological response to a specific pathogen. No study to date has combined both factors. The present studies were carried out with an immunologically manipulated mouse model of IBD. Mice homozygous for the severe combined immunodeficiency (*scid*) mutation develop IBD with adoptive transfer of $CD4^+$ T cells expressing high levels of CD45RB ($CD45RB^{high}$ $CD4^+$ T cells). These mice do not develop IBD in germfree conditions, implicating undefined intestinal flora in the pathogenesis of lesions. In controlled duplicate studies, the influence of a single murine pathogen, *Helicobacter hepaticus*, in combination with the abnormal immunological response on the development of IBD was assessed. The combination of *H. hepaticus* infection and $CD45RB^{high}$ $CD4^+$ T-cell reconstitution resulted in severe disease expression similar to that observed in human IBD. This study demonstrates that IBD develops in mice as a consequence of an abnormal immune response in the presence of a single murine pathogen, *H. hepaticus*. The interaction of host immunity and a single pathogen in this murine system provides a novel model of human IBD, an immunity-mediated condition triggered by bacterial infection.

Inflammatory bowel disease (IBD) is chronic inflammation limited to the large bowel (ulcerative colitis) or anywhere in the gastrointestinal tract (Crohn's disease). The etiopathogenesis of IBD is poorly understood; however, two general hypotheses exist (15). First, the disease is due to an abnormal and uncontrolled immune response to luminal antigen, and second, the disease is initiated by an appropriate immune response to an enteric pathogen, which is still unidentified.

Recently developed models of IBD have focused on aberrant regulation of the immune response in rodents, including cytokine (10, 20) or T-cell-receptor mutant mice (12) and *scid* mice reconstituted with $CD4^+$ T cells (3, 16). These animals develop severe colitis when maintained in conventional conditions. IBD does not develop in animals which are rederived into germfree conditions (20, 21), which clearly implicates enteric flora in disease pathogenesis. It is evident from these studies that in the presence of aberrant immune regulation, enteric flora plays a major role in the pathogenesis of IBD; however, the specific component of the microflora which triggers the disease process has not been identified. These findings agree with human clinical observations that suggest a role for bacterial flora in IBD; antibiotic therapy with metronidazole or bypassing the fecal stream are effective in maintaining disease remission after surgery (19, 27).

Enteric flora in described models of IBD has not been characterized, and in theory, a potential pathogen could exist in the flora (4). The aim of these studies was to test the hypothesis that IBD develops as a consequence of an abnormal immune response in the presence of a single enteric pathogen.

The murine pathogen used in these studies was *Helicobacter hepaticus* (6, 25), which is related to the human pathogen *Helicobacter pylori*. *H. hepaticus* causes persistent hepatitis and hepatocellular carcinoma (7, 25) in certain strains of mice and recently has been associated with spontaneous proliferative colitis in immunodeficient mice (26) and in germfree mice monoinfected with *H. hepaticus* (8). *H. hepaticus* infection is endemic in many commercial and academic mouse colonies (22).

MATERIALS AND METHODS

To achieve our aim, a previously described model of IBD was utilized involving severe combined immunodeficient (*scid*) mice, which lack immunocompetent B and T cells reconstituted with congenic $CD4^+$ T cells expressing high levels of CD45RB ($CD45RB^{high}$ $CD4^+$ T cells) (11, 16, 17). Enteric flora has been implicated in the pathogenesis of this model (21). Germfree status does not facilitate the assessment of the contribution of normal gut flora to the disease process; therefore, to assess the influence of a single pathogen in the presence of normal mucosal flora, the mice used in these studies were colonized with a well-characterized, defined flora (altered Schaedler formula) (14) consisting of eight anaerobic species of bacteria which represent the major flora of barrier-reared rodents.

Animals. All animal experiments were approved by the institutional committee on animal care. The mice were housed in autoclaved polycarbonate microisolator cages on autoclaved bedding and fed autoclaved pelleted diet and autoclaved water ad libitum. All animal manipulations were carried out in a laminar-flow hood. The mice were monitored for contamination by exogenous microflora every 2 weeks as follows. Samples of feces, bedding, water, and feed were suspended in sterile phosphate-buffered saline (PBS). Samples were plated onto Columbia agar with 5% sheep blood (Remel Labs, Lenexa, Kans.), blood agar (Remel Labs), tryptic soy broth (TSB; Remel Labs), and nutrient agar with dextrose (Difco Laboratories, Detroit, Mich.) and incubated in microaerobic, anaerobic, and aerobic conditions at both 37°C and room temperature. The plates were assessed for growth after 24 h and 1 week of incubation. At necropsy, mice were assessed for *Salmonella* spp., *Escherichia coli*, *Citrobacter* spp., and protozoan infection.

Experimental design. In each of two experiments, 20 *H. hepaticus*-free *scid* C.B-17 mice with defined flora (Taconic Farms) were used. The mice were screened for infection with *Helicobacter* spp. as described previously (22) and for

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TABLE 1. Combination of altered immune function and *H. hepaticus* infection results in severe clinical presentation and gross lesions

Study group	No. of animals (*n*)			
	Total	With rectal prolapse	Euthanatized due to clinically severe disease	With grossly thickened colon, cecum, and rectum on necropsy
Control				
Study 1	4	0	0	0
Study 2	4	0	0	0
CD45RB^{high} T cells alone				
Study 1	4	0	0	1
Study 2	4	0	1	1
H. hepaticus alone				
Study 1	6	1	0	1
Study 2	6	0	0	1
CD45RB^{high} T cells and *H. hepaticus*[a]				
Study 1	6	4	3	5
Study 2	6	2	3	6

[a] There were significant associations (as assessed by chi-square test) between the combination of both CD45RB^{high} T cells and *H. hepaticus* infection and animals with rectal prolapse ($P < 0.01$), animals euthanized due to clinically severe disease ($P < 0.01$), and animals with grossly thickened colon, cecum, and rectum on necropsy ($P < 0.0005$).

adventitious agents. After the initial screening period of 2 weeks, the mice were assigned to one of four study groups on the basis of CD45RB^{high} CD4$^+$ T-cell reconstitution and infection with *H. hepaticus* (see Table 1) as follows: group 1 ($n = 4$), control; group 2 ($n = 4$), reconstituted with CD45RB^{high} CD4$^+$ T cells alone; group 3 ($n = 6$), infected with *H. hepaticus* alone; group 4 ($n = 6$), infected with *H. hepaticus* and reconstituted with CD45RB^{high} CD4$^+$ T cells. Mice were monitored daily for signs of disease and assessed for weight loss. Those with severe rectal prolapse or a weight loss of >20% were euthanized. The remaining mice were euthanatized at 8 weeks and 12 weeks postreconstitution in studies 1 and 2 (see below), respectively. The study duration was based on previous studies using this model (16, 17).

Infection with *H. hepaticus* and reconstitution with CD45RB^{high} CD4$^+$ T cells. Mice were infected with *H. hepaticus* (ATCC 51448). *H. hepaticus* was grown under microaerobic conditions at 37°C in serum-free TSB (Difco) for 72 h. The bacteria were harvested, resuspended in TSB to a concentration of approximately 10^7 CFU of *H. hepaticus* per ml as assessed by optical density, and visualized by phase-contrast microscopy to assess motility and morphology (8). The inocula were assayed for contamination by culture on Columbia agar under aerobic and anaerobic conditions. The mice were dosed by oral gavage on three alternate days. Control mice and group 2 mice received sham inoculation with sterile TSB. *H. hepaticus* infection was confirmed by fecal culture and by PCR (22). Two weeks after successful infection with *H. hepaticus*, mice were reconstituted with CD45RB^{high} CD4$^+$ T cells from BALB/c mice as described previously (16, 17) with a single 100-μl intraperitoneal injection of cells at a concentration of 2×10^6 cells/ml. Reconstitution was confirmed by flow cytofluorography of splenocytes by use of a technique described previously (16, 17). Control mice and mice in group 3 received a single intraperitoneal injection of sterile PBS.

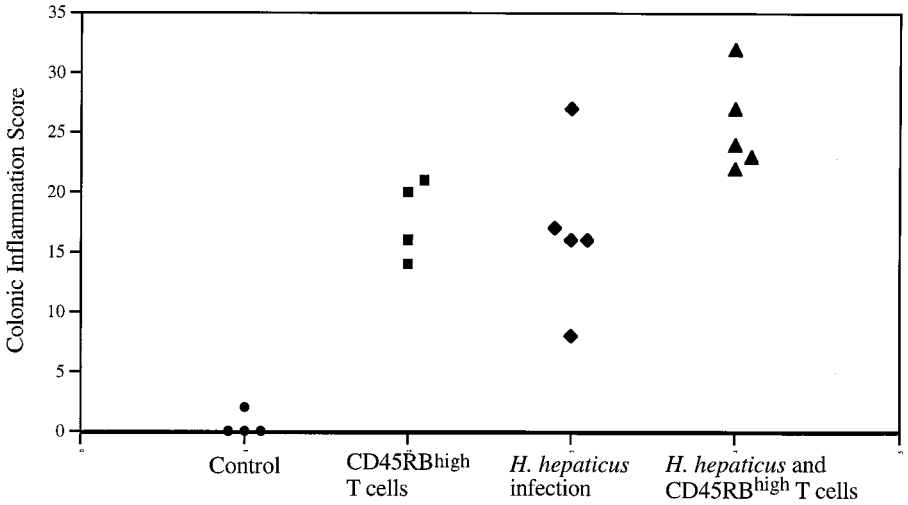

FIG. 1. Increased severity of colonic inflammation in mice infected with *H. hepaticus* and abnormal immune response (study 1). Mice reconstituted with CD45RB^{high} CD4$^+$ T cells and infected with *H. hepaticus* had colonic inflammation similar to that seen in human IBD. The inflammation was more severe than that in controls or in animals with either factor alone. The scores for colonic inflammation were as follows: the control median score, 0 (individual scores, 0, 0, 0, and 2); the median score for CD45RB^{high} T-cell reconstitution alone, 18 (individual scores, 14, 16, 20, and 21); the median score for *H. hepaticus* infection alone, 16 (individual scores, 8, 16, 16, 17, and 27); and the median score for *H. hepaticus* infection and CD45RB^{high} T-cell reconstitution, 24 (individual scores, 22, 23, 24, 27, and 32). The colonic inflammation scores of mice both infected with *H. hepaticus* and reconstituted with CD45RB^{high} CD4$^+$ T cells were significantly higher ($P < 0.05$) than those of controls or mice with either factor alone as assessed by nonparametric analysis. The lesions were graded mild, moderate, and severe with a score of 1 to 3, respectively. This lesion severity score was then multiplied by the score for lesion distribution, which was as follows: 1, small focal lesions; 2, large focal or infrequent multifocal lesions; 3, frequent multifocal lesions; 4, coalescing multifocal or diffuse lesions. Inflammation was scored separately for the distal, middle, and proximal regions of the colon, and the results were combined. Inflammation was scored on the basis of leukocytic cellular infiltration and fibrosis-acellular infiltration. The potential maximum colonic inflammation score was 72.

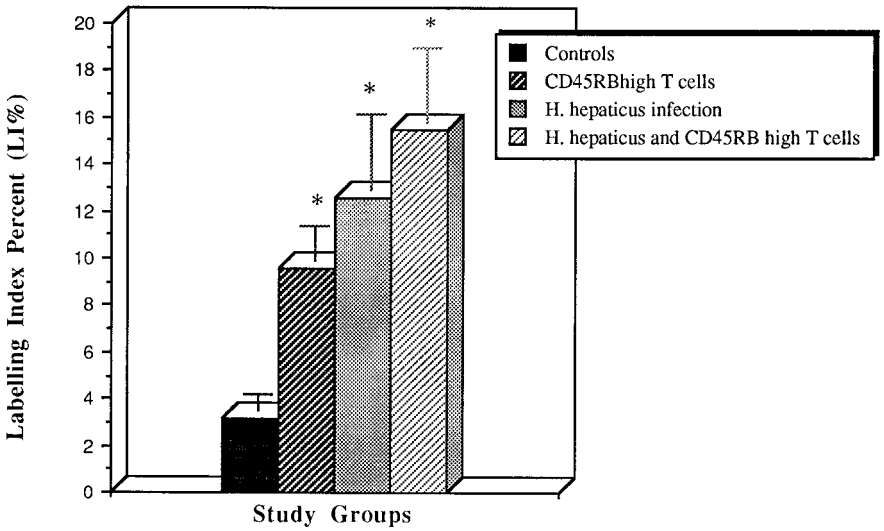

FIG. 2. Increased colonic epithelial cell proliferation associated with the combination of abnormal immune function and infection with *H. hepaticus*. Reconstitution with CD45RB^{high} CD4$^+$ T cells and infection with *H. hepaticus* either alone or in combination resulted in a significantly higher colonic epithelial cell proliferation compared to that of controls (*, $P < 0.01$). The combination of both factors resulted in an increase in epithelial cell proliferation higher than that of either factor alone. All results are expressed as LI% (mean ± standard deviation) and are as follows: for controls ($n = 3$), 3.2% ± 0.7%; for group with CD45RB^{high} T-cell reconstitution alone ($n = 3$), 9.6% ± 1.5%; for group with *H. hepaticus* infection alone ($n = 4$), 12.6% ± 3.2%; for group with CD45RB^{high} T-cell reconstitution and *H. hepaticus* infection ($n = 6$), 15.5% ± 3.1%. Epithelial cell proliferation was assessed by BrdU incorporation (19). Epithelial cell proliferation was quantified as the LI%, which is calculated as the number of labeled cells divided by the total number of cells in a colonic crypt expressed as a percentage. A mean of 10 entire crypts were assessed from each of three sites, adjacent to the rectum, the distal colon, and the proximal colon. Total colonic epithelial cell proliferation was the combined mean LI% of all three sites.

Histopathology. The entire gastrointestinal tract was examined for gross lesions. The colon, rectum, and small intestine were fixed by the Swiss roll technique, which allowed histological visualization of the entire length of the intestine (13). At necropsy, gastrointestinal and liver tissues were fixed in either Carnoy's fixative or neutral buffered 10% formalin, processed by standard methods, embedded in paraffin, and sectioned at thicknesses of 5 μm (formalin) and 4 μm (Carnoy's). Tissues were visualized with hemotoxylin-and-eosin and Warthin-Starry stains. The gastrointestinal and liver lesions were assessed for gross and histological lesions. Histological assessment of the cecum and colon included grading of epithelial lesions (goblet cell depletion and epithelial hyperplastic changes) and inflammation (degree of stromal and cellular inflammation).

Colonic epithelial cell proliferation assessed by BrdU immunohistochemistry. Animals received a single intraperitoneal injection of bromodeoxyuridine (BrdU; 50 mg/kg) from a freshly made stock solution of 5 mg/ml dissolved in PBS. The mice were sacrificed 1 h later. Tissue sections for assessment of BrdU incorporation were fixed in Carnoy's fixative. Incorporation of BrdU was assessed on 4-μm-thick sections by use of a previously described avidin-biotin immunohistochemical technique (9) with a monoclonal antibody against BrdU (Dakopatts, Glostrup, Denmark). The entire length of colonic mucosa was available for assessment of BrdU incorporation. Only colonic crypts visible in their entire length were analyzed. A mean of 10 crypts were assessed in each of three areas: immediately adjacent to the rectum, the distal colon, and the proximal colon. BrdU incorporation is expressed as the labeling index percent (LI%), which is calculated by expressing the number of labeled cells (cells that incorporated BrdU) as a percentage ratio of the total number of cells in the crypt. The mean LI% for the three sites was the LI% for the total colon.

RESULTS

All mice experimentally infected with *H. hepaticus* had positive fecal cultures for *H. hepaticus*. Infection with *H. hepaticus* was confirmed by PCR. Mice with defined flora not experimentally infected with *H. hepaticus* remained free of the bacteria. Reconstitution with CD45RB^{high} CD4$^+$ T cells was confirmed for all mice injected with cells (data not shown). The mice remained free of contamination with other enteric pathogens or exogenous microflora for the duration of the study.

Disease presentation. Mice presenting with morbidity associated with severe wasting (weight loss of >20%) or rectal prolapse were sacrificed before the study end points (Table 1). Control mice and mice infected with *H. hepaticus* alone did not present with clinically severe symptoms. One mouse (12.5%) reconstituted with CD45RB^{high} CD4$^+$ T cells alone was euthanatized prior to the study end point. Fifty percent of mice reconstituted with CD45RB^{high} CD4$^+$ T cells and infected with *H. hepaticus* had clinically severe disease prior to the end of the study and were killed.

Gross lesions. No gross lesions were apparent in the controls. Two mice infected with *H. hepaticus* had thickening of the colon or cecum (Table 1). Two mice reconstituted with CD45RB^{high} CD4$^+$ T cells alone had thickening of the cecum and colon. Eleven of the 12 mice reconstituted with CD45RB^{high} CD4$^+$ T cells and infected with *H. hepaticus* (91.6%) had gross lesions with thickening of the large bowel evident. The thickening was observed in 33% of the mice in the rectum only, in 25% of the mice in the cecum and colon, in 8% of the mice in the cecum alone, and in 17% of the mice in the colon alone. The thickened mucosa was observed throughout the large bowel and distal small intestine in one animal (8%).

Histopathology. (i) Study 1. In the first study at 8 weeks postreconstitution, *scid* mice with defined flora reconstituted with CD45RB^{high} CD4$^+$ T cells developed inflammatory lesions of mild to moderate intensity. *scid* mice with defined flora infected with *H. hepaticus* alone also developed IBD, and the lesions were less severe than in those reconstituted with CD45RB^{high} CD4$^+$ T cells alone. The combination of *H. hepaticus* infection and reconstitution with CD45RB^{high} CD4$^+$ T cells resulted in the most severe manifestation of both clinical disease and histological lesions in comparison to the other study groups. Colonic inflammation lesion scores were significantly higher in mice reconstituted with CD45RB^{high} CD4$^+$ T cells and infected with *H. hepaticus* ($P < 0.05$) than in mice with either factor alone or in controls (Fig. 1). Inflammation was typically characterized by a multifocal to coalescing, mixed mononuclear cell infiltrate which manifested both histiocytic

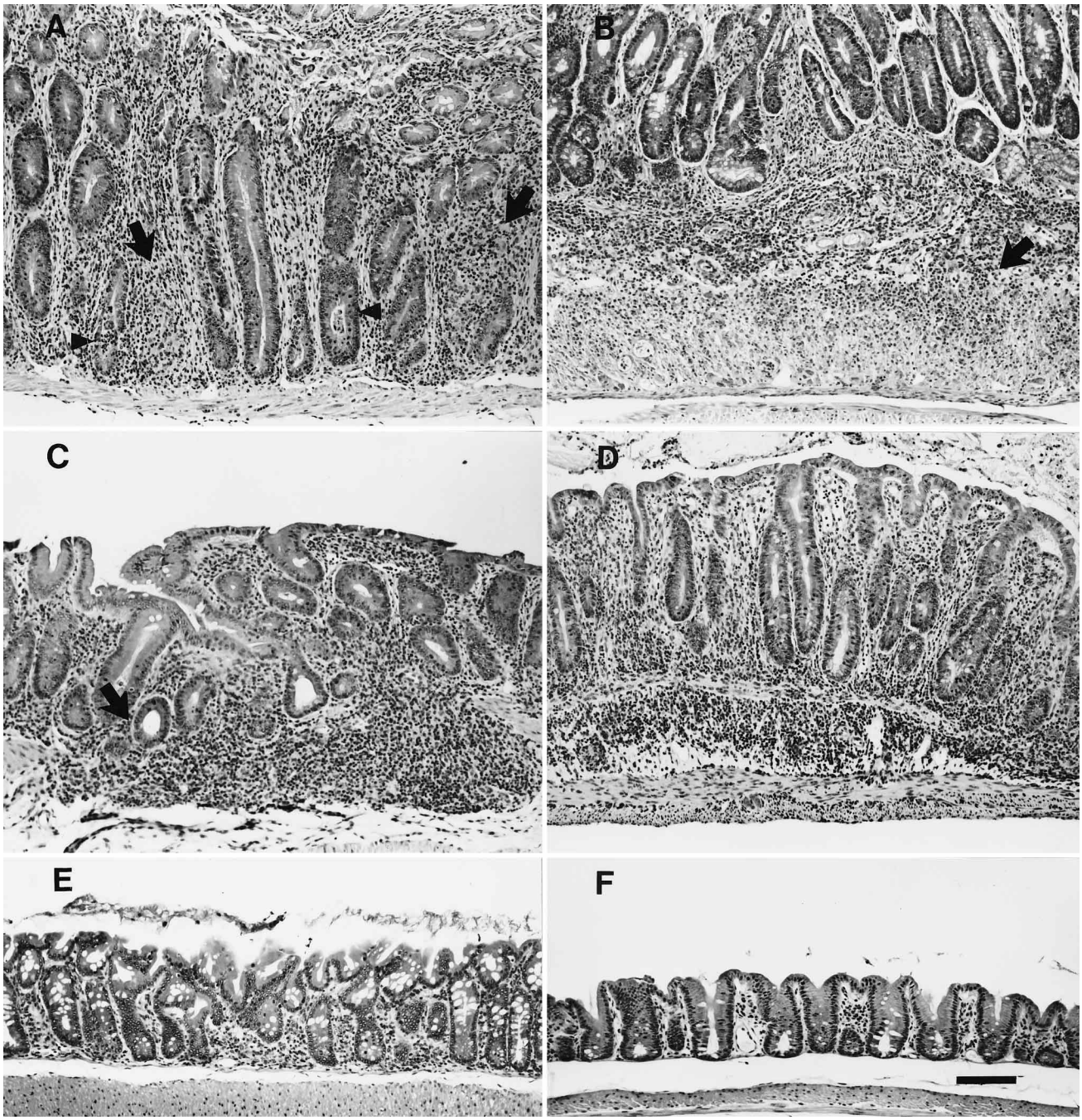

FIG. 3. Infection with *H. hepaticus* and reconstitution with CD45RB^{high} CD4^{+} T cells of *scid* mice result in a severe disease similar to human IBD (study 2). *scid* mice reconstituted with CD45RB^{high} CD4^{+} T cells and infected with *H. hepaticus* have severe cecal and colonic inflammation and mucosal hyperplasia. The lesions identified in these mice were similar to those described for human IBD. (A to D) Histological lesions identified in mice reconstituted with CD45RB^{high} CD4^{+} T cells and infected with *H. hepaticus*. Intense lymphohistiocytic and multifocal granulomatous inflammation (A, arrows) in the colonic mucosa and submucosa with multifocal transmural inflammation (B, arrow) are visible. Transmural inflammation was manifested in the cecum and/or colon of four of the six mice in the group reconstituted with CD45RB^{high} CD4^{+} T cells and infected with *H. hepaticus* in study 2. The hyperplastic crypt epithelium is characterized by elongation of crypts, diminished goblet cell differentiation, focal necrosis, and crypt abscess formation (A, arrowheads) and infiltration of epithelial crypts into the submucosa (C, arrow). Similar lymphohistiocytic inflammation and hyperplastic changes are visible in the cecum (D). (E and F) Negative infection controls. Colonic (E) and cecal (F) mucosa from *scid* mice reconstituted with CD45RB^{high} CD4^{+} T cells but not infected with *H. hepaticus* are shown. Mucosal inflammation and hyperplasia are typically of mild to moderate intensity; however, one of the four mice included in the study group manifested transmural inflammation in the colon. Bar, 100 μm (applies to all panels).

and lymphocytic morphology, associated with mild focal erosion of the mucosal epithelium. Epithelial cell proliferation as assessed by BrdU incorporation paralleled the changes observed in mucosal hyperplasia (Fig. 2). Lesions in the cecum were similar to those in the colon, with mucosal hyperplasia being more pronounced. A lack of gross or histological lesions in the defined-flora control group indicated that the defined enteric flora was insufficient to induce IBD.

(ii) Study 2. The dramatic difference in IBD presentation among different experimental groups observed in study 1 was also observed in study 2. However, the longer duration of study 2 was associated with progression to more severe lesions. Again, the colonic inflammation and hyperplasia were of greatest intensity in mice reconstituted with CD45RB^{high} CD4$^+$ T cells and infected with *H. hepaticus*. Mice reconstituted with CD45RB^{high} CD4$^+$ T cells and infected with *H. hepaticus* had thickening of the large intestine, with severe histological lesions consisting of severe chronic inflammation with mononuclear cell infiltration extending into the submucosa, focal transmural inflammation, and mucosal damage, including crypt abscesses, fibrosis, and epithelial necrosis characteristic of lesions associated with human IBD. The lesions observed in the mice encompass features of both ulcerative colitis and Crohn's disease. Representative histopathological lesions are shown in Fig. 3.

DISCUSSION

This is the first study that clearly demonstrates that the development of severe IBD and the associated lesions in mice with aberrant immune function, similar to the human condition, requires the presence of a single pathogenic bacterial species. In this model, both an abnormal immunological response and an enteric pathogen contribute to the development of IBD, which provides a valuable system for the investigation of these two factors in the disease process. The role of infectious agents in the initiation of human IBD has been suspected; however, despite intensive research, an infectious agent that is consistently associated with either Crohn's disease or ulcerative colitis has not been identified. Human and animal intestines are colonized with an enormously complex microflora composed of a large variety of indigenous aerobic and anaerobic bacteria, making the identification of a single putative pathogen difficult. This study provides a useful system to investigate the role of specific bacterial pathogens in the etiology of IBD.

Since the discovery of *H. pylori* and its association with chronic gastritis and gastric carcinoma, interest in other *Helicobacter* species has increased. As with *H. pylori*, *H. hepaticus* causes a persistent infection with chronic inflammation, both in the liver and in the lower bowel (7, 8). IBD is characterized by chronic inflammation similar to gastritis caused by *H. pylori*. Preliminary evidence from our laboratory suggests that both pathogens have virulence factors that may be associated with severe inflammation and cancer. Although to date most research has focused on *H. pylori* infection in humans, evidence from animal studies and immunocompromised patients suggests that other *Helicobacter* species exist in the large bowel. Several nongastric *Helicobacter* spp. have been reported to infect immunocompetent and immunocompromised humans, infection has been associated with gastroenteritis (1, 2, 23), and in the case of two species, *Helicobacter cinaedi* and *Helicobacter fennelliae*, human infection has been associated with colitis and proctitis (5, 24). Although the evidence indicates that intestinal *Helicobacter* spp. can colonize humans and elicit disease, the specific role of these intestinal pathogens in human IBD has not been elucidated.

The results of this study may extend beyond the field of IBD. For example, infective agents are suspected in many immunity-mediated diseases such as rheumatoid arthritis and psoriasis (18). Although many bacterial species have been implicated in these conditions, all are capable of eliciting an inflammatory response. Our study describes a model which can dissect the selective roles of both immune response regulation and infection in induction and progression of disease and may provide the means to investigate other disease processes.

In conclusion, the absence of severe, clinically significant IBD in immunocompromised mice with defined flora implicates an intestinal pathogen in the pathogenesis of disease. Our findings demonstrate that in combination with aberrant immune response regulation, normal flora plus the presence of a single species of pathogenic bacteria, *H. hepaticus*, is sufficient to produce severe clinically apparent IBD. In the same way that the discovery of *H. pylori* revolutionized our understanding of duodenal ulcers, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma, the identification that a single pathogenic bacteria can dramatically influence the outcome of IBD may reconceptualize our experimental approach to dissecting mechanisms operable in induction of IBD. Future research regarding the role of *Helicobacter* spp. in the etiopathogenesis of IBD will broaden our understanding of this complex, debilitating disorder affecting millions of humans worldwide.

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