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A Novel Model of Inflammatory Bowel Disease: Mice Deficient for the Multiple Drug Resistance Gene, mdr1a, Spontaneously Develop Colitis

Chetan M. Panwala,* Jon C. Jones,† and Joanne L. Viney‡†

The murine multiple drug resistance (mdr) gene, mdr1a, encodes a 170-kDa transmembrane protein that is expressed in many tissues including intestinal epithelial cells, a subset of lymphoid cells and hematopoietic cells. We report that mdr1a knockout (mdr1a<sup>−/−</sup>) mice are susceptible to developing a severe, spontaneous intestinal inflammation when maintained under specific pathogen-free animal facility conditions. The intestinal inflammation seen in mdr1a<sup>−/−</sup> mice has a pathology similar to that of human inflammatory bowel disease (IBD) and is defined by dysregulated epithelial cell growth and leukocytic infiltration into the lamina propria of the large intestine. Treating mdr1a<sup>−/−</sup> mice with oral antibiotics can both prevent the development of disease and resolve active inflammation. Lymphoid cells isolated from mice with active colitis are functionally reactive to intestinal bacterial Ags, providing evidence that there is enhanced immunologic responsiveness to the normal bacterial flora during IBD. This study is the first description of spontaneous colitis in a gene knockout mouse with an apparently intact immune system. This novel model of spontaneous colitis may provide new insight into the pathogenesis of IBD, the nature of dysregulated immune reactivity to intestinal bacterial Ags, and the potential functional role of mdr genes expressed in the cells and tissues of the colonic microenvironment. The Journal of Immunology, 1998, 161: 5733–5744.

The inflammatory bowel diseases (IBD)<sup>2</sup>, Crohn’s disease and ulcerative colitis, are syndromes characterized by chronic inflammation of the gastrointestinal tract. The etiology of these disorders remains unknown despite many years of investigation. The current hypotheses suggest that persistent intestinal inflammation may be the result of either enhanced or aberrant immunologic responsiveness to normal constituents of the gut lumen (1, 2) or an overall autoimmune dysregulation and imbalance (3–11). The recent establishment of various animal models relevant for studying the pathogenesis of intestinal inflammation has provided some insight into potential disease mechanisms. In particular, the discovery that many animals with altered T cell populations or cytokine deficiencies spontaneously develop colitis has implicated an immune imbalance as one of the critical factors in the manifestation of IBD (3–11). Although the pathologic changes associated with IBD and the extensive lymphoid infiltrates into the inflammatory lesion suggest that there is some immunologic dysregulation, it is likely that many of the immunologic changes associated with IBD are probably not the primary cause of the disease process, but may be due to secondary nonspecific inflammation.

There is increasing evidence that the intestinal microflora is an important cofactor in the pathogenesis of intestinal inflammation. The development of spontaneous colitis in many mouse models can be prevented if mice are generated and maintained in a germ-free environment or treated with oral antibiotics, highlighting the central role for bacterial colonization in the initiation and/or perpetuation of experimental IBD (4, 12–16). There is also evidence that human patients with IBD suffer from adverse and enhanced reactivity to their autologous resident intestinal flora (1, 2, 17). Considering that the cells of the mucosal immune system are protected from the large antigenic load in the gut lumen by a single layer of epithelial cells, it seems reasonable to assume that epithelial cells play a major role in providing a barrier that regulates contact between bacteria and immune cells. In support of this, transgenic mice expressing a mutant cadherin on their epithelial cells in the small intestine spontaneously develop IBD, presumably due to breaches occurring within the epithelial cell barrier (18). Thus, proteins expressed in epithelial cells may in some way contribute, directly or indirectly, to maintaining the protective barrier.

Intestinal epithelial cells and some lymphocyte subsets have been shown to express multiple drug resistance (MDR) genes (19–24). These genes belong to a family of transporters known as ATP binding cassette transporters, which are characterized by their ability to pump small amphiphilic and hydrophobic molecules across membranes in an ATP-dependent manner (25, 26). MDR genes were first identified by their ability to confer resistance to chemotherapeutic agents in tumors and neoplastic cell lines (27). Three MDR genes have been identified in rodents and two in humans, each of which has a restricted pattern of tissue expression and, most likely, a different function. Although it is well known that some of these gene products (MDR1 in humans and mdr1a and mdr3 in mice) can actively pump toxic drugs out of cells, their natural in vivo role has not been fully elucidated (28, 29). In mice, mdr1a is expressed on many tissues including intestinal epithelial cells, CD8<sup>+</sup> T cells, a subset of CD4<sup>+</sup> T cells, hematopoietic cells, and cells at the blood-brain barrier (19, 21, 23, 30, 31). The function of mdr1a in each of these different cell types and tissues is unknown.
Recently, mice with a targeted deletion of the mdr1a gene were generated (32). Analysis of these knockout mice has revealed that they have an increased sensitivity to certain drugs, but do not appear to show any constitutive abnormalities (31–34). In the current study we observe that mdr1a−/− mice housed under specific pathogen-free (SPF) conditions develop spontaneous intestinal inflammation. We describe the nature of the inflammatory cell infiltrate, describe a role of the intestinal microflora in the initiation and perpetuation of colitis in these mice, and provide evidence that the development of colitis appears to arise as a result of a defect in the intestinal epithelial barrier.

Materials and Methods

**Mice**

FVB control mice and mdr1a−/− mice were initially purchased from Tacorny (Germantown, NY) and then bred and maintained in the SPF facility at Immunix (Seattle, WA) in accordance with approved ethical guidelines. mdr1a−/− mice had been backcrossed onto the FVB strain for at least seven generations at Tacorny Farms before shipping. Control and knockout mice used for experiments were age matched and received treatment simultaneously. Mice were monitored on alternate days for the presence of diarrhea and mucous discharge from the anus. All mice were bred and maintained under SPF conditions in Thoren isolation racks under positive pressure and fed autoclaved food and water ad libitum. The quality of the SPF facility is monitored through the Charles River Health Monitoring Plus program (Charles River, Wilmington, MA) using the Charles River tracking profile. Sentinel mice receiving bedding from donor cages are tested for viral, parasitic, and bacterial infection. There were no viruses, parasitides, or pathogenic bacteria detected in our colony throughout the course of the study. Of particular note, we did not detect any evidence of Helicobacter infection (Helicobacter bilis, Helicobacter hepaticus, or Helicobacter spp.) in any mice.

**Treatment of mice with oral antibiotics**

Streptomycin sulfate (1.25% w/v), neomycin (1% w/v), bacitracin (1% w/v), and amphotericin (0.25% w/v) were dissolved in autoclaved drinking water (pH 2.5–2.8) supplemented with saccharine (1% w/v) and then filtered through a 0.22-μm Nalgene filter (Nalgene, Milwaukee, WI). As a control, mice received saccharine alone (1% w/v) in the drinking water. All reagents were obtained from Sigma (St. Louis, MO). Regular drinking water was replaced with antibiotic or saccharine drinking water during treatment.

**Antibodies**

The following primary Abs were used for flow cytometric analysis and immunoperoxidase histochemistry: anti-CD4 (rat IgG; L3T4), anti-CD8α (rat IgG; 53-6.7), anti-TCRβ (hamster IgG; H57-597), anti-TCRγδ (hamster IgG; GL3), anti-GR1 (rat IgG; RB6–8C5), anti-B220 (CD45R) (rat IgG; RA3–6B2), and anti-CD3e (hamster IgG; 500A2; Immunex). The primary Abs were conjugated either with biotin or R-phycocerythrin (PE). All Abs were obtained from PharMingen (San Diego, CA) except where indicated. The following secondary Abs were used for immunoperoxidase histochemistry: biotinylated sheep anti-rat IgG (Amersham, Arlington Heights, IL) and biotinylated goat anti-hamster IgG (Caltag, South San Francisco, CA).

**Flow cytometric analysis of isolated cells**

Isolated cells were incubated with the appropriate PE- or biotin-conjugated primary Ab for 30 min on ice in PBS supplemented with 5% BSA and 0.02% sodium azide essentially as described previously (35). Stained cells were washed two times, incubated with streptavidin-allophycocyanin (Molecular Probes, Eugene, OR), and then fixed in 1% paraformaldehyde before analysis. Stained cells were analyzed on a Becton Dickinson FACScan flow cytometer (Immunocytochemistry Systems, San Jose, CA) using Becton Dickinson CellQuest software.

**Rhodamine efflux assay**

Lymphocytes were isolated from mice and incubated with 0.4 μg/ml rhodamine 123 (Sigma) for 15 min at 37°C in complete RPMI 1640 supplemented with 10% FCS. For two-color analysis, cells were simultaneously stained with PE-conjugated primary Abs. The stained, rhodamine-loaded cells were then washed two times and incubated in complete medium without out rhodamine 123 for an additional 2 h at 37°C. Cells were then washed two times in complete medium and analyzed on a Becton Dickinson FACScan flow cytometer (Immunocytochemistry Systems) using Becton Dickinson CellQuest software.

**Immunoperoxidase histochemistry analysis of frozen sections**

Large intestines were removed, embedded in OCT compound (Tissue-Tek, Torrence, CA), frozen in a dry ice/ethanol bath, and stored at −80°C until use. Frozen sections (5 μm) of intestine were cut, fixed for 15 min in acetone, and stored at −80°C until staining. Peroxidase staining was performed using the Vectastain ABC system (Vector Laboratories, Burlingame, CA) according to specified directions as described previously (35). Brieﬂy, tissue sections were preincubated in 5% goat serum, 5% bovine serum, and 1% mouse serum for 30 min at room temperature and then incubated with biotin-conjugated primary Ab for 1 h at room temperature. Slides were washed three times in Tris-buffered saline, further incubated with avidin-peroxidase (Vectastain ABC system), washed, and finally incubated with diaminobenzidine substrate (Sigma) before counterstaining in hematoxylin. Positive cells were identiﬁed by the presence of a brown reaction product.

**Immunofluorescence analysis of frozen sections**

Tissues were frozen in liquid nitrogen as described above, and 5-μm cryosections were cut, air dried, and acetone fixed before staining. Tissue sections from both mdr1a+/− and control FVB mice were stained with either 20 μg/ml FITC-conjugated anti-MDR mAb (C219; Signet Laboratories, Dedham, MA) or FITC-conjugated IgG2a isotype control mAb (PharMingen) in the presence of 2 mM TOTO-3 (Molecular Probes) and 30% mouse serum. Sections were visualized using a confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA).

**Intraepithelial lymphocyte (IEL) and lamina propria lymphocyte (LPL) cell isolations**

IEL were isolated according to a modiﬁcation of published procedures (35, 36). Brieﬂy, the small and large intestines were removed and washed in Ca2+/Mg2+-free HBSS (Life Technologies, Bethesda, MD). Peyer’s patches were removed, and the intestines were opened longitudinally, cut into 0.5-cm pieces, and placed in 1 mM EDTA in Ca2+/Mg2+-free HBSS at 37°C for three sequential 15-min incubations with intermittent vortexing to remove epithelial cells and IEL. Supernatants were collected and pooled for IEL puriﬁcation. The remaining tissues were then washed in PBS and incubated in trypsin-EDTA two times to remove any residual epithelial cells, and the denuded tissues were digested with 90 U/ml collagenase type VIII (Sigma) in RPMI 1640/10% FCS for three sequential 20-min incubations at 37°C with intermittent vortexing to release LPL. Supernatants were collected and pooled for LPL puriﬁcation. IEL and LPL were puriﬁed by passing cells over a prewet glass wool column before spinning them through a 40%/80% discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) as previously described (35). The cells at the 40%/80% interface were collected, washed, and resuspended in complete medium for analysis.

**Intestinal bacterial Ag preparation**

Intestinal bacterial Ags were prepared from fecal contents harvested from the cecum according to a modiﬁcation of published procedures (37). Brieﬂy, individual preparations were processed from three different mouse sources: 1) an FVB control mouse, 2) an mdr1a−/− mouse with no signs of inﬂammation, and 3) an mdr1a−/− mouse with active colitis. Theecal contents from the individual mice were placed in a 2-mL bead shaker tube half-ﬁlled with 2-μm zirconium beads suspended in PBS. The mixture was vortexed in a bead beater (BioSpec Products, Bartlesville, OK) for 30 s at 9000 rpm and placed on ice. The intestinal bacterial slurry was then microcentrifuged at 14,000 × g, and the supernatant was harvested and ﬁltered through a 0.22 μm ﬁlter. The relative protein content in each sample was estimated using a Bradford assay (Bio-Rad, Hercules, CA).

**Proliferation assay**

Cell suspensions were prepared by gently mashing mesenteric lymph nodes (MLN) between ground glass slides and passing them through nylon mesh. MLN cells were cultured at a density of 2 × 105 cells/well in 96-well flat-bottom plates in complete RPMI supplemented with 10% FCS in a ﬁnal volume of 200 μL in a humidified 5% CO2 incubator at 37°C for 48 h. Cells were cultured either in the presence of titrating amounts of intestinal bacterial Ag preparations, in the presence of 1 μg/ml Con A, or in...
FIGURE 1. Mucosal lymphocytes from FVB mice, but not mdr1a<sup>-/-</sup> mice, exhibit mdr1a functional activity. MLN cells (A) and mucosalIEL and LPL (B) were loaded with rhodamine 123, simultaneously stained with PE-conjugated mAb (to CD3, CD4, CD8, TCR<sub>β</sub>, or TCR<sub>γδ</sub>), and then allowed to efflux rhodamine for 2 h. Cells were then analyzed by flow cytometry, and mdr1a activity was determined by decreased rhodamine intensity. Axes show log<sub>10</sub> fluorescence intensity measured at 525 nm for rhodamine 123 and 575 nm for PE after excitation with a 488-nm argon laser. The FACS profiles represent data from one of two separate experiments.

Serum Ab titers
Mice were exsanguinated, and the serum was collected. Polyclonal serum Ab titers were determined by isotype-specific ELISAs for IgA, IgE, IgG1, IgG2a, IgG2b, IgG3, and IgM as previously described (38). Briefly, 96-well Nunc Maxisorp plates (Nunc, Naperville, IL) were coated overnight at 4°C with isotype-specific anti-mouse Ig Abs (0.2 mg/well), blocked with PBS/10% FCS for 2 h at room temperature, and washed with PBS/0.1% Tween-20. Serum samples and control samples were serially diluted in PBS/10% FCS starting at 1:100. Plates were incubated for 2 h at room temperature, washed, and incubated with the appropriate peroxidase-conjugated specific anti-isotype detecting Ab for an additional 2 h at room temperature. Plates were washed again, and enzyme activity was detected using TMB microwell peroxidase substrate reagents (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and 2 N H<sub>2</sub>SO<sub>4</sub>. The amount of reaction product was assessed on an ELISA plate reader at OD 450 nm using the Deltasoft program (DeltaPoint, Monterey, CA). The affinity-purified Abs used for determining isotype-specific serum Ab titers by ELISA were obtained from Southern Biotechnology Associates (Birmingham, AL), except for anti-IgE, which was obtained from Serotec (Oxford, U.K.), and anti-IgG2a, which was obtained from PharMingen. Ig concentrations were determined by comparing test sample dilution curves with known concentrations of isotype controls. Results are indicated as mean concentration ± 1 SD of two separate mice for each group.

Bone marrow chimeric mice
mdr1a<sup>-/-</sup> mice and FVB mice were irradiated with 10 Gy using a <sup>137</sup>Cs source, rested for 4–6 h, and then reconstituted by i.v. injection with 2 × 10<sup>9</sup> bone marrow cells collected from the femurs of appropriate donors.

Bacterial identification
Analysis of cecal and colonic bacteria (Phoenix Laboratories, Everett, WA). Mice were killed under sterile conditions, and colonic washout samples were harvested and shipped to Microcheck for bacterial identification by microbial cellular fatty acid analysis. Samples were subcultured at Microcheck, and microbial fatty acid profiles were determined by high-resolution gas chromatography. Identification of bacteria in individual samples was determined by comparing the fatty acid profiles of the unknown sample with known profiles from microbial fatty acid libraries.

Results
mdr1a is functionally expressed by mucosal lymphocytes and is on the apical surface of colonic epithelial cells
Efflux of rhodamine 123 from preloaded lymphocytes has been used to demonstrate the presence of mdr activity in peripheral T cells (19, 30). However, the assay does not distinguish whether rhodamine efflux is due to mdr1a activity, mdr3 activity, or perhaps even another unidentified transporter. We therefore analyzed rhodamine 123 efflux in lymphocytes from normal FVB mice and mdr1a<sup>-/-</sup> mice to determine whether rhodamine 123 efflux was associated with a functional mdr1a gene and to identify which mucosal and peripheral lymphocyte subsets might exhibit the pump activity. mdr1a-associated functional activity was detected in MLN cells from normal FVB mice but not from mdr1a<sup>-/-</sup> mice, as determined by ability to efflux rhodamine 123 (Fig. 1A). These data indicate that a functional mdr1a gene product is necessary for rhodamine 123 efflux. To determine which subsets of mucosal lymphocytes have the mdr1a activity, we performed the rhodamine efflux assay on IEL and LPL isolated from the large intestines of FVB mice. We simultaneously stained these cells with mAbs against CD3, CD4, CD8, TCR<sub>β</sub>, and TCR<sub>γδ</sub> (Fig. 1B). The individual CD3, CD4, CD8, TCR<sub>β</sub>, and TCR<sub>γδ</sub> subsets within the IEL and LPL compartments had similar efflux profiles. Virtually all of the CD8<sup>+</sup> cells and TCR<sub>γδ</sub><sup>+</sup> cells appeared to exhibit mdr activity and efflux rhodamine 123, whereas only half of the CD4<sup>+</sup> cells and half of the TCR<sub>β</sub><sup>+</sup> cells were able to efflux the dye. None of the mucosal lymphocyte populations from the mdr1a<sup>-/-</sup> mice were able to efflux the rhodamine 123 (data not shown), further suggesting that mdr1a is necessary for rhodamine 123 efflux activity in mucosal lymphocytes.
Immunofluorescent staining was used to localize mdr1a expression in the colon. Fig. 2 shows mdr1a staining (green) on transverse sections of colons from FVB control (+/+) and mdr1a-deficient (−/−) mice. The sections were counterstained with TOTO-3 (red) to visualize gut architecture. mdr1a staining appears to be restricted to the apical surface of epithelial cells in FVB mice. No mdr1a staining was apparent on sections from mdr1a−/− mice or on sections from FVB mice stained with an isotype control (data not shown). We were also unable to detect any mdr1a staining on LPL or on IEL in FVB mice, despite functional evidence that these cells express mdr1a (see above). It is unclear whether this was due to low expression levels of mdr1a on mucosal lymphocytes or to the relatively low frequency of lymphocytes within each section.

Clinical characteristics and incidence of colitis in mdr1a−/− mice

Approximately 20–25% of the 169 mdr1a knockout mice included in this part of the study appeared to develop loose stools and anal mucous discharge by 1 year of age (Table I). Histologic analysis of mice with clinical signs of colitis confirmed the presence of active intestinal inflammation that was primarily restricted to the large intestine. The difference in incidence between males (20%) and females (26%) was not significant. The average age at onset was approximately 20 wk, although the first signs of colitis development could be seen in mice ranging between 8 and 36 wk of age. Although some mice with colitis developed a wasting-type disease, the majority of colitic animals were maintained for up to 3 mo without signs of cachexia. Disease was nontransmissible, since mdr1a−/− mice both with and without colitis were housed in the same cage. We observed no clinical or histologic signs of colitis in

![Image](https://via.placeholder.com/150)

**FIGURE 2.** mdr1a is expressed on the apical surface of the intestinal epithelial cells in the colon. Frozen sections of large intestine from FVB control mice (+/+ ) and mdr1a-deficient mice (−/−) were stained for mdr1a expression with anti-mdr FITC mAb C219 (green) and counterstained with TOTO-3 (red). The scale bars represent 20 μm. The staining shown is representative of two mice analyzed. Similar staining was seen in two separate experiments.

<table>
<thead>
<tr>
<th>Age at Disease Onset (wk)</th>
<th>5–10</th>
<th>11–15</th>
<th>16–20</th>
<th>21–25</th>
<th>26–30</th>
<th>31–35</th>
<th>Total</th>
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<tr>
<td>Male</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>16/82</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>23/87</td>
<td></td>
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*a* Colitis was determined by the presence of loose stools and mucous discharge from the anus.

*b* Incidence of colitis was monitored in mdr1a−/− mice housed under SPF conditions.

*c* Number of mice with colitis.

*d* There was no significant difference in incidence of colitis between males and females at a 95% confidence interval (z = .092).
any FVB control mice, heterozygous mdr1a<sup>+/−</sup> mice, or other immunocompetent mouse strains of any age housed within the facility.

**Histopathologic changes associated with colitis in mdr1a<sup>−/−</sup> mice**

The severe inflammation of the large intestine that develops in mdr1a<sup>−/−</sup> mice bears resemblance to the human IBD, ulcerative colitis. Histopathologic analysis of multiple colitic mdr1a<sup>−/−</sup> animals revealed inflammation spread along the entire length of the colon. The intestinal inflammation was characterized by a massive thickening of the mucosa and evidence of inflammatory cell infiltrates into the lamina propria in mdr1a<sup>−/−</sup> mice with active colitis (Fig. 3a) compared with mdr1a<sup>−/−</sup> mice without colitis (Fig. 3b).

**FIGURE 3.** mdr1a<sup>−/−</sup> mice develop severe spontaneous inflammation of the large intestine. Frozen sections stained with hematoxylin and eosin revealed a massive thickening of the colonic tissues when mdr1a<sup>−/−</sup> mice with established colitis (a) were compared with mdr1a<sup>−/−</sup> mice without colitis (b); original magnification, ×63.5. c, Crypt abscesses and ulcers extending through the mucosa to the muscularis were prevalent in many sections of intestine from mdr1a<sup>−/−</sup> mice with active colitis (original magnification, ×250). d, Evidence of dysregulated epithelial cell growth in colitic mice (original magnification, ×500). a–d are from individual mice and are representative of multiple tissue sections analyzed from >15 mice per group.
Occasional crypt abscesses and ulcerations extending through the mucosa to the muscular layer were also often noted in the colitic animals (Fig. 3c). Crypt length was greatly increased in colitic animals, with approximately three- to fivefold more epithelial cells per crypt in colitic mice (Fig. 3a) compared with noncolitic mice (Fig. 3b). There also appeared to be some evidence of dysregulated epithelial cell growth in colitic mdr1a^{−/−} mice in areas of inflammation (Fig. 3d).

Immunohistochemical analysis of the gastrointestinal tissues of animals with prevalent colitis or animals with early stages of colitis was used to further characterize the lymphocytic infiltrate into the mucosa of the large intestine compared with control FVB mice (Fig. 4). There was an increased number of infiltrating CD3^{+} T cells scattered diffusely throughout the lamina propria of inflamed colonic tissue from mice with active colitis (Fig. 4a) compared with colonic tissue from control FVB mice (Fig. 4c). A less dramatic CD3^{+} T cell infiltrate was observed in mice with early colitis (Fig. 4b).

FACS analysis of the large-intestine IEL and LPL isolated from colitic animals revealed that the majority of the infiltrating T cells were CD4^{+}TCRαβ^{+} (Table II). Although there was an apparent decrease in the relative proportion of CD8^{+} T cells in the lamina propria of colitic animals, the absolute number of CD8^{+}TCRαβ^{+} T cells was not diminished, probably owing to the fourfold increase in the total number of LPL isolated from colitic animals (data not shown). There was no apparent increase in TCRγδ^{+} T cells in the inflamed tissue (data not shown).

In addition to T cell infiltrates, immunohistochemical analysis also revealed the presence of numerous B220^{+} B cell clusters/follicles within the lamina propria of animals with active colitis (Fig. 4d) compared with control FVB animals (Fig. 4f). Whereas the T cells were diffusely scattered throughout the lamina propria, the B cell infiltrates appeared to be localized to follicular clusters within the mucosa. The follicular clusters varied in size and were only observed in the colon. In contrast to animals with fully developed colitis, animals with intermediate colitis mostly had individual B220^{+} cells scattered throughout the lamina propria.
throughout the lamina propria and the few clusters that were present were reduced in size (Fig. 4e).

Gr1⁺ cellular infiltrates were also prominent throughout the lamina propria of the large intestine in mice with active colitis (Fig. 4g) compared with control FVB mice, which appeared devoid of Gr1⁺ cells (Fig. 4i). The Gr1⁺ infiltrates were uniformly present throughout the lamina propria, although an increased number of cells did appear to be migrating across the epithelium into the gut lumen in regions where the epithelial barrier may have been compromised (Fig. 4g). Mice with

Table II.  *Mucosal T cell subsets isolated from colitic and control intestines*[

<table>
<thead>
<tr>
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<th>IEL</th>
<th>LPL</th>
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<tr>
<td></td>
<td>Colitic <em>mdrla</em> /⁻/⁻ mice</td>
<td>Control FVB mice</td>
</tr>
<tr>
<td>% CD4⁺/CD8⁻</td>
<td>42</td>
<td>10</td>
</tr>
<tr>
<td>% CD8⁺/CD4⁻</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td>% CD4⁺/CD8⁺</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>% TCRαβ⁺</td>
<td>94</td>
<td>50</td>
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</table>

IEL and LPL were isolated from large intestines from *mdrla* /⁻/⁻ mice with active colitis and from age-matched control FVB mice and stained for CD4, CD8, and TCRαβ expression. Numbers are expressed as a percent of total mononuclear cells analyzed.

FIGURE 5. Treating *mdrla* /⁻/⁻ mice with oral antibiotics can prevent the onset of colitis. Seventy *mdrla* /⁻/⁻ mice with no visible signs of colitis were selected for the study. Regular drinking water was replaced with antibiotics and saccharine dissolved in drinking water (n = 31) or saccharine alone dissolved in drinking water (n = 39). Mice were monitored on alternate days for clinical signs of colitis for up to 16 wk. Mice were then killed and analyzed for histopathologic signs of colitis. Data represent the percentage of mice with clinical or histopathologic evidence of colitis.

FIGURE 6. Therapeutic antibiotic treatment can reverse the gut inflammation associated with established colitis in *mdrla* /⁻/⁻ mice. Mice with active signs of colitis (loose stools and anal discharge) were placed on antibiotic treatment for 10 wk (n = 3). At the end of the treatment period, colons were removed from untreated *mdrla* /⁻/⁻ mice with active colitis (a), control FVB mice (b), and *mdrla* /⁻/⁻ mice therapeutically treated with antibiotics (c). Note that the colon from the antibiotic-treated mouse does not appear inflamed and shows little residual evidence of thickening. d, Immunohistochemical analysis of colonic tissues from mice treated with antibiotics showed persistence of CD3⁺ T cells despite significant reduction in inflammation.
Therapeutic antibiotic treatment can reverse ongoing active and did not show any other overt signs of intestinal disease. Focal region of inflammatory cell accumulation (data not shown) two different methods (see Materials and Methods). All of the mice had a mixed anaerobic flora with heavy growth of Bacteroides sp.

**Prophylactic treatment with oral antibiotics can prevent spontaneous colitis in mdr1a<sup>−/−</sup> mice**

It has been well established in a majority of mouse models that spontaneous colitis can be prevented if mice are housed under germfree conditions, suggesting that the presence of the gut flora is necessary for the development of colitis (4, 12, 14). It has also been previously demonstrated in another model of spontaneous colitis that treating mice with antibiotics can prevent inflammation (13). In the first part of the study, we treated mdr1a<sup>−/−</sup> mice with broad-spectrum oral antibiotics to eliminate the commensal gut flora and then monitored for the development of colitis. Control mice were given water treated with saccharine alone. None of the mice had detectable colitis at the beginning of the study.

The incidence of colitis in mdr1a<sup>−/−</sup> mice treated with antibiotics was greatly reduced compared with mdr1a<sup>−/−</sup> mice treated with saccharine alone (Fig. 5). In the 16-wk treatment, 14 of 31 saccharine-treated mdr1a<sup>−/−</sup> mice developed colitis (loose stools and mucous discharge), whereas only 1 of 39 antibiotic-treated mdr1a<sup>−/−</sup> mice developed any signs of inflammation (no clinical signs, histopathologic evidence only). The one antibiotic-treated mdr1a<sup>−/−</sup> mouse that showed signs of colitis had only one minor focal region of inflammatory cell accumulation (data not shown) and did not show any other overt signs of intestinal disease.

**Therapeutic antibiotic treatment can reverse ongoing intestinal inflammation in mdr1a<sup>−/−</sup> mice**

To ascertain whether the intestinal inflammation in mice with active colitis could be reversed by antibiotic treatment, mice with active colitis (as determined by evidence of loose stools and mucous discharge from the anus) were therapeutically treated with antibiotics in the drinking water. Within 3 wk of continuous antibiotic therapy, the previously colitic mice had no residual visible signs of anal discharge or loose stools. To determine whether the pathology associated with colitis could also be reversed along with the gross symptoms, mice treated with antibiotics for 10 wk were killed and the colons analyzed histopathologically. As expected, the colons of untreated colitic animals were grossly thickened (Fig. 6a), compared with control animals (Fig. 6b). Interestingly, the gross thickening of the colon associated with active IBD was significantly reduced in mice that had received therapeutic antibiotic treatment (Fig. 6c). On further histopathologic examination of these intestines, we observed little evidence of inflammation, and the general gut architecture appeared similar to that of control mice (data not shown). Immunohistochemical analysis revealed that the mice treated with antibiotics had no evidence of persisting Gr1<sup>+</sup> infiltrates or B cell follicles (data not shown). However, despite the apparent reduction in inflammation arising from antibiotic treatment, there still appeared to be an increased number of CD3<sup>+</sup> T cells in the lamina propria of these animals (Fig. 6d) compared with control mice (Fig. 4c).

**Analysis of bacterial flora in colitic and control mice**

Although the commensal bacterial flora resident in the intestinal lumen of the gut appears to play a major role in the induction of colitis, little is known about the role of individual bacteria in the

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**Table III. Identification of bacterial species**

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<thead>
<tr>
<th>Mice</th>
<th>Aerobic Bacteria</th>
<th>Frequency$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVB</td>
<td>Lactobacillus sp.</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>Strep species, α-hemolytic</td>
<td>1/8</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>1/8</td>
</tr>
<tr>
<td></td>
<td>Enterococcus sp.</td>
<td>2/8</td>
</tr>
<tr>
<td></td>
<td>Bacillus sp.</td>
<td>1/8</td>
</tr>
<tr>
<td></td>
<td>β hemolytic bacillus</td>
<td>2/8</td>
</tr>
<tr>
<td></td>
<td>Enterococcus</td>
<td>1/8</td>
</tr>
<tr>
<td></td>
<td>Proteus mirabilis</td>
<td>3/8</td>
</tr>
<tr>
<td>mdr1a&lt;sup&gt;−/−&lt;/sup&gt; (no colitis)</td>
<td>Lactobacillus sp.</td>
<td>6/8</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>5/8</td>
</tr>
<tr>
<td></td>
<td>Bacillus sp.</td>
<td>1/8</td>
</tr>
<tr>
<td></td>
<td>β hemolytic bacillus</td>
<td>1/8</td>
</tr>
<tr>
<td></td>
<td>Enterococcus</td>
<td>2/8</td>
</tr>
<tr>
<td></td>
<td>Proteus mirabilis</td>
<td>2/6</td>
</tr>
<tr>
<td>mdr1a&lt;sup&gt;−/−&lt;/sup&gt; (active colitis)</td>
<td>Proteus mirabilis</td>
<td>2/6</td>
</tr>
<tr>
<td></td>
<td>Enterococcus</td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td>Staphylococcus sp.</td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td>Lactobacillus sp.</td>
<td>4/6</td>
</tr>
<tr>
<td></td>
<td>Bacillus sp.</td>
<td>2/6</td>
</tr>
</tbody>
</table>

$^a$ Samples from cecum and colon were analyzed for identification of bacteria by two different methods (see Materials and Methods). All of the mice had a mixed anaerobic flora with heavy growth of Bacteroides sp.

$^b$ Frequency of mice with identified bacteria.
munocompromised mice can give rise to a pathology resembling plus the SD of two separate animals analyzed for each experimental group. Values are expressed as increase in serum Ab titers of all isotypes compared with noncolitic and control mice. Serum Ab titers were similar in noncolitic and control mice.

We observed a significant increase in proliferation in response to bacterial Ags when MLN from mdr1a−/− mice with active colitis were compared with those of other mice (p < 0.05). In contrast, there was no statistically significant difference in the response to bacterial Ag stimulation when MLN from FVB mice were compared with MLN from noncolitic mdr1a−/− mice (p > 0.1). Reactivity profiles of each source of MLN were similar regardless of the source of bacterial Ags (data not shown). No significant differences were noted in the proliferative responses to Con A or anti-CD3 between any of the three groups of mice analyzed in these experiments (p > 0.1). The data shown are from one experiment and are representative of three similar experiments.

**TABLE IV. Incidence of colitis in bone marrow chimeric mice**

<table>
<thead>
<tr>
<th>Host</th>
<th>BM Donor</th>
<th>% of Mice Developing Colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>mdr1a−/−</td>
<td>FVB</td>
<td>25 (n = 12)</td>
</tr>
<tr>
<td>FVB</td>
<td>mdr1a−/−</td>
<td>0 (n = 40)</td>
</tr>
<tr>
<td>mdr1a−/−</td>
<td>mdr1a−/−</td>
<td>100 (n = 15)</td>
</tr>
<tr>
<td>FVB</td>
<td>FVB</td>
<td>0 (n = 20)</td>
</tr>
</tbody>
</table>

Bone marrow chimeric mice were generated as described in Materials and Methods. Mice were observed for both clinical and histopathological signs of colitis.

**FIGURE 8.** Serum Ab titers are elevated in mdr1a−/− mice with active colitis. Isotype-specific ELISAs were performed on serum from mdr1a−/− mice with active colitis (mdr1a−/−, colitis), mdr1a−/− mice without active colitis (mdr1a−/−), and control FVB animals (FVB). Colitic mice had an increase in serum Ab titer of all isotypes compared with noncolitic and control mice.

initiation and perpetuation of intestinal inflammation. In this study we analyzed the bacterial flora present in the cecum and colon of mdr1a−/− mice with colitis, mdr1a−/− mice with no evidence of active colitis, and control FVB mice. Table III lists the major bacterial species recovered from the animals. All of the mice appeared to have a gut bacterial profile devoid of any pathogenic organisms.

Recently, it has been shown that Helicobacter infection in immunocompromised mice can give rise to a pathology resembling colitis (39–41). To ensure that colitis in the mdr1a−/− mice was not due to Helicobacter infection, samples from colitic, noncolitic, and control mice were analyzed by PCR for the presence of H. bilis, H. hepatus, and Helicobacter spp. All mice were negative (data not shown).

*In vitro proliferative reactivity of cells from the gut-associated lymphoid tissue following stimulation with intestinal bacterial Ags*  

Since bacterial colonization of the intestine appears to be crucial for the initiation of spontaneous colitis in mdr1a−/− mice, we analyzed the in vitro proliferative response of cells isolated from the MLNs following stimulation with intestinal Ags (Fig. 7). Intestinal Ags were prepared from cecal contents obtained from FVB control mice, mdr1a−/− mice with no overt signs of colitis, and mdr1a−/− mice with active colitis. MLN were removed from the same animals, and cell suspensions were tested for proliferative reactivity against each of the intestinal bacterial Ag preparations.

We observed a significant increase in proliferation in response to bacterial Ags when MLN from mdr1a−/− mice with active colitis were compared with those of other mice (p < 0.05). In contrast, there was no statistically significant difference in the response to bacterial Ag stimulation when MLN from FVB mice were compared with MLN from noncolitic mdr1a−/− mice (p > 0.1). Reactivity profiles of each source of MLN were similar regardless of the source of bacterial Ags (data not shown). No significant differences were noted in the proliferative responses to Con A or anti-CD3 between any of the three groups of mice analyzed in these experiments (p > 0.1). The data shown are from one experiment and are representative of three similar experiments.

**Spontaneous colitis correlates with a lack of mdr1a expression on intestinal epithelial cells**  

Given that mdr1a is expressed on both lymphocytes and epithelial cells in the large intestine, we sought to determine whether the spontaneous development of colitis was the result of an mdr1a defect within the lymphoid or within the epithelial cell population. We generated irradiation bone marrow chimeras in which irradiated FVB animals were reconstituted with bone marrow from mdr1a−/− or FVB donors, or irradiated mdr1a−/− mice were reconstituted with bone marrow from FVB or mdr1a−/− donors. As controls, additional groups of mice were generated in which FVB mice were reconstituted with FVB bone marrow and mdr1a−/− mice were reconstituted with mdr1a−/− bone marrow. The incidences of colitis in the groups are listed in Table IV. mdr1a−/− mice that were reconstituted with wild-type bone marrow developed signs of colitis within 12 wk after reconstitution. After being maintained for 52 wk, 25% of the mice had developed active colitis. In contrast, two groups of FVB mice, which were reconstituted with mdr1a−/− bone marrow, were maintained for up to 38 wk without any overt signs of colitis. As expected, the control FVB mice reconstituted with FVB bone marrow did not develop colitis. Surprisingly, 100% of the mdr−/− mice reconstituted with mdr1a−/− bone marrow developed colitis. The reason for the increased incidence in the latter group compared with unmanipulated mdr1a−/− mice is unclear, although it is possible that radiation damage to the mucosa sufficiently enhanced susceptibility to developing colitis.
Discussion

Mucosal immunology has advanced in recent years with the discovery of several different mouse models of IBD. Most of the mouse models described involve a disruption or modification of the immune system. Spontaneous colitis occurs in mice genetically deficient for TCRαβ⁺ cells (5) or the cytokines IL-2 (6) or IL-10 (4) and in mice deficient for the Go2, signaling protein (42). In addition, IBD occurs in human CD3⁺-transgenic mice reconstituted with normal bone marrow (7), in HLA-B27 transgenic rats (43), and in immunodeficient SCID mice reconstituted with CD4⁺CD45RB⁺ cells (9, 12, 44). The great diversity of these models implies that there are complex mechanisms regulating the various components of the mucosal immune system that create and maintain a homeostatic balance. Disruption of this balance in any number of ways can obviously lead to the development of an adverse inflammatory response.

Here we describe a novel model of spontaneous colitis occurring in mdr1a⁻/⁻ mice that is characterized by a broad inflammatory response that appears restricted to the entire length of the colon. The histopathology of the inflammation is most consistent with features associated with ulcerative colitis in humans and is characterized by dysregulated epithelial cell growth, ulcerations, crypt abscesses, and lymphocytic infiltration into the mucosa. Given the large number and diverse nature of the IBD models, it is not surprising that there is some controversy regarding the precise nature of the cells responsible for initiating and perpetuating IBD. The diffuse lymphocytic infiltrates in the mdr1a⁻/⁻ mice are primarily CD4⁺TCRαβ⁺ T cells and Gr1⁺ cells. In addition, follicular B cell clusters are prominent throughout the mucosa.

mdr1a⁻/⁻ mice require a commensal bacterial flora for the development of colitis. Colitis can be prevented in mdr1a⁻/⁻ animals by oral treatment with broad-spectrum antibiotics to eliminate intestinal bacteria. The requirement for bacterial flora in the induction of colitis in mdr1a⁻/⁻ mice is consistent with many of the other IBD models. IBD does not develop in the TCRα or IL-10 knockout animals maintained under germfree conditions (4, 14), and IBD can be prevented in the CD4⁺CD45RB⁺ SCID transfer model if the animals have a reduced number of enteric flora or are treated with broad-spectrum antibiotics (12, 13). In addition to preventing the initiation of IBD, we show here that antibiotic treatment can therapeutically reverse colitis in mdr1a⁻/⁻ mice with active inflammation. This indicates that intestinal flora is not only necessary for initiating inflammation but is also required for sustaining the inflammatory response. Interestingly, despite the reversal of inflammation, a large number of CD3⁺ T cells remain present in the mucosa. The functional state of these T cells is unclear.

In the course of this study, we observed a relatively high incidence of colitis in mdr1a⁻/⁻ mice treated with saccharine compared with unmanipulated knockout mice in the colony. We have no definitive explanation for this, since there is no a priori reason why saccharine should make animals more susceptible to developing colitis. However, it is possible that changes do occur in the commensal flora when saccharine is included in the drinking water, perhaps permitting outgrowth of certain bacterial species. Despite the increased incidence of colitis in the saccharine-treated mice, the addition of antibiotics was sufficient to eliminate the problem and prevent both the development of colitis and reverse ongoing intestinal inflammation.

It is possible that an individual bacterial species that colonizes the intestine might be responsible for initiating inflammation in these murine IBD models. To determine whether there might be a specific bacterial pathogen associated with disease in the mdr1a⁻/⁻ mice, we analyzed the bacterial composition of the resident cecal and colonic flora in colitic and noncolitic mdr1a⁻/⁻ mice and control FVB mice. We were not able to identify a single bacterial species that could be solely responsible for triggering intestinal disease. There did appear to be variations in the relative abundance of certain bacterial species in the different samples analyzed, and while it remains possible that an excess of a singular species might be responsible for the initiation of colitis, this may simply reflect a selective advantage for certain bacteria in the local microenvironment during intestinal inflammation.

Another potential explanation for the requirement of intestinal flora in the induction of IBD is that cross-reactive epitopes may exist between intestinal flora and enterocytes, such that the enterocytes become the target of an autoimmune inflammatory response. In support of this, IBD in TCRα knockout mice is accompanied by the appearance of autoAbs, including those directed against epithelial cell proteins (8, 45). mdr1a⁻/⁻ mice with active colitis have large B cell follicles in the lamina propria. These mice also have a massive increase in serum Ab titers of all isotypes, suggesting a polyclonal activation of B cells; however, it is unclear whether the Ab response is directed against intestinal tissues. We are currently breeding the mdr1a⁻/⁻ mice with B cell-deficient mice to determine whether B cell activation is a critical component in the initiation of colitis.

Since data from many of the other IBD models indicate that T cells are a major component in the induction of IBD, and much of the intestinal damage in IBD appears to be the result of T cell-mediated injury (46, 47), we decided to analyze the functional activity of T cells isolated from mdr1a⁻/⁻ mice with active colitis. Lymphocytes isolated from animals with active inflammation appeared to be significantly more responsive to intestinal bacterial Ags compared with control animals. Lymphocytes isolated from mdr1a⁻/⁻ mice without active inflammation had a similar proliferative reactivity compared with control FVB animals. This indicates that the mdr1a defect does not inherently cause hyperresponsiveness to bacterial Ags. Rather, it appears that lymphocytes become hyperresponsive to bacterial stimulation only after the onset of inflammation. The finding that bacterial Ags prepared from animals with colitis and animals without colitis were equally potent in stimulating proliferation further suggests that colitis is probably not caused by a single pathogenic organism.

A central question in the mdr1a⁻/⁻ mouse IBD model is, where does the defect that leads to colitis reside? mdr genes are expressed on a number of immune and nonimmune cell types in mice and humans (19–21, 23). Thus, it is possible that mdr1a may have several different functional activities depending on the site of expression. We show here that mdr1a is expressed on murine peripheral and mucosal T lymphocytes including all TCRβ⁺ and CD8⁺ cells and a proportion of TCRαβ⁺ and CD4⁺ cells. Despite the lack of mdr1a expression on immune cells in the mdr1a⁻/⁻ mice, the immune system does not appear to have any gross phenotypic or functional defects. It is known that mdr1a is also expressed on the apical surface of colonic epithelial cells, where it has been shown to actively pump orally administered toxins into the lumen of the gut (48). There is also evidence that mdr1a may play a role in Cl⁻ channel regulation in epithelial cells (49–51). Thus, we thought it possible that mdr1a⁻/⁻ mice have functional defects within the intestinal epithelial barrier that might ultimately lead to colitis. To assess whether immune or epithelial cell defects were responsible for the susceptibility to colitis in mdr1a⁻/⁻ mice, we generated a series of chimeric mice. Irradiated mdr1a⁻/⁻ mice reconstituted with wild-type bone marrow spontaneously developed colitis. In contrast, irradiated wild-type mice reconstituted with mdr1a-deficient bone marrow cells did not develop colitis.
These data clearly suggest that the defect that leads to the initiation of colitis in mdr1a-deficient mice is concordant with defective mdr1a expression on a radiation-resistant element within the host, most likely the epithelial cells lining the gut. These data also suggest that the colitis in mdr1a<sup>-/-</sup> mice is not likely to be associated with intact immune cell deficiencies.

The precise mechanistic defect causing colitis in the mdr1a<sup>-/-</sup> mice is unclear. One possibility is that bacterial products/toxins become sequestered within epithelial cells, since small amphiphilic, hydrophobic molecules are less likely to be secreted in the absence of a functional mdr pump. It is not unreasonable to assume that a buildup of bacterial products within mdr1a-defective epithelial cells could subsequently initiate an inflammatory response, either by direct toxicity to the epithelial cell or indirectly by altering the nature of the Ag-presenting capability of the epithelial cell. The demonstrated requirement for bacterial flora in the initiation and progression of the inflammation, and the association of disease with defective mdr1a expression in epithelial cells, suggests that one of these scenarios might be true. We are currently attempting to ascertain the precise function of mdr1a in colonic epithelial cells.

With all of the different immune models of colitis, it has become apparent that the intestinal microenvironment is an exquisitely regulated system and that perturbation of individual components within this environment can lead to inflammation. The model described here has many similarities with existing murine models of spontaneous colitis, including an inflamed pathology restricted to the colon and a dependence on intestinal flora. In contrast to most spontaneous murine models of colitis, the transplantation of intestinal lymphocytes from mice colonized by transfer of CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells to SCID recipients. J. Immunol. 158:3464.

Morrissey, P. J., and K. Charrier. 1994. Induction of wasting disease in SCID mice by the transfer of normal CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells and the regulation of this autoreactivity by CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells. Eur. Immunol. 145:357.


