Tumorigenesis and Neoplastic Progression

Dual Infection with *Helicobacter bilis* and *Helicobacter hepaticus* in P-Glycoprotein-Deficient *mdr1a*^{-/-} Mice Results in Colitis that Progresses to Dysplasia

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Patients with inflammatory bowel disease (IBD) are at increased risk for developing high-grade dysplasia and colorectal cancer. Animal IBD models that develop dysplasia and neoplasia may help elucidate the link between inflammation and colorectal cancer. *Mdr1a*^{-/-} mice lack the membrane efflux pump p-glycoprotein and spontaneously develop IBD that can be modulated by infection with *Helicobacter* sp: *H. bilis* accelerates development of colitis while *H. hepaticus* delays disease. In this study, we determined if *H. hepaticus* infection could prevent *H. bilis*-induced colitis. Unexpectedly, a proportion of dual-infected *mdr1a*^{-/-} mice showed IBD with foci of low- to high-grade dysplasia. A group of dual-infected *mdr1a*^{-/-} animals were maintained long term (39 weeks) by intermittent feeding of medicated wafers to model chronic and relapsing disease. These mice showed a higher frequency of high-grade crypt dysplasia, including invasive adenocarcinoma, possibly because *H. hepaticus*, in delaying the development of colitis, allows time for transformation of epithelial cells. Colonic epithelial preparations from co-infected mice showed increased expression of *c-myc* (5- to 12-fold) and interleukin-1 α / β (600-fold) by real-time polymerase chain reaction relative to uninfected wild-type and *mdr1a*^{-/-} animals. This animal model may have particular relevance to human IBD and colorectal cancer because certain human MDR1 polymorphisms have been linked to ulcerative colitis and increased risk for colorectal cancer. (*Am J Pathol* 2005, 166:1793-1806)

Colorectal cancer is one of the most common forms of cancer in industrialized countries and is increasing in incidence.¹ Although animal models have been useful in understanding the mechanisms involved in the pathogenesis of colorectal cancer, differences between these models and human disease may limit their relevance.² For example, targeted disruption in the mouse of the *APC* gene, which has been implicated in human colon cancer, results primarily in cancer of the small intestine³ suggesting that additional animal models would be useful to study cancer development in the colon.

Patients with inflammatory bowel disease (IBD) have an increased risk for developing colorectal cancer.^{4,5} Hence, the induction of inflammation and its subsequent link to dysplasia and cancer needs further study. Animal models that display inflammation of the large bowel, which progresses to dysplasia and neoplasia would be helpful in understanding the molecular and cellular mechanisms that result in the transformation of large intestinal epithelial cells during colon carcinogenesis. *Helicobacter* are microaerophilic gram-negative spiral bacterial organisms⁶ that have been associated with gastric cancer in humans,⁷ and as well as hepatitis,⁸ hepatocellular carcinoma,⁹ and IBD in rodents.¹⁰ In particular, there is established literature implicating *H. pylori* with gastric cancer in man,¹¹ and *H. hepaticus* has been associated with hepatocellular carcinoma in A/JCr mice¹² and colorectal cancer in RAG2-knockout mice.^{13,14} We have previously used *H. hepaticus* and *H. bilis* infections in genetically susceptible strains of mice such as IL-10^{-/-} and TCR α ^{-/-}¹⁵ and in *mdr1a*^{-/-}¹⁶ to understand microbial modulation of IBD. *Mdr1a*^{-/-} mice lack the transmembrane efflux transporter, p-glycoprotein, and develop spontaneous colitis.¹⁷ We have previously shown

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that *H. bilis* accelerates and increases the severity of inflammation and hyperplasia in *mdr1a*^{-/-} mice, while *H. hepaticus* delays the development of colitis.¹⁶ Therefore, we hypothesized that *H. hepaticus* infection might delay or ameliorate the development of *H. bilis*-induced colitis. Notably, we found that dual-infection with both *H. hepaticus* and *H. bilis* in *mdr1a*^{-/-} mice resulted in colitis that was characterized by dysplasia, particularly if longer survival could be attained with intermittent treatment of infected mice with medicated wafers. Dysplastic colonic tissue from dual-infected *mdr1a*^{-/-} mice had increased numbers of CD4⁺ T cells, macrophages, and Cox-2⁺ cells. Colonic epithelial cell preparations from dual-infected *mdr1a*^{-/-} mice had increased expression of the proinflammatory cytokines interleukin (IL)-1 α and IL-1 β , and the oncogene *c-myc*. This animal model may be useful to study factors important in the progression of chronic inflammation to dysplasia and could have relevance to human bowel disease and colorectal cancer because certain MDR1 polymorphisms in man have been linked to ulcerative colitis¹⁸ and increased risk for neoplasia¹⁹ including colorectal cancer.²⁰

Materials and Methods

Mice

For study 1, *mdr1a*^{-/-} (FVB.129P2-PA Abcb1a^{tm1Bor}) were obtained from Taconic Farms (Albany, NY) and 3- to 6-week-old female *mdr1a*^{-/-} specific pathogen-free mice were used. These mice have a targeted mutation in *Pgy3* and the gene name has now been changed from *Pgy3* to *Abcb1a*, ATP-binding cassette, subfamily B (MDR/TAP), member 1A. For study 2 and study 3, colony mice were obtained from our breeder *mdr1a*^{-/-} and FVB wild-type mouse colony (originally obtained from Taconic Farms). All mice were certified free of *Helicobacter* spp. by the vendor and retested on site by fecal polymerase chain reaction (PCR) before each experiment. Animals were housed in a specific pathogen-free facility at the University of Washington in polycarbonate microisolator or ventilated cages containing Bed-O-Cob (Andersons, Maumee, OH) and nestlets. Mice were fed irradiated Picolab Rodent Diet 20 no. 5053 (PMI Nutrition Int., Brentwood, MO) and autoclaved, acidified water. All supplies entering animal rooms were autoclaved and rooms were maintained at 21 to 23°C, 45 to 55% humidity, with 28 air changes/hour 12/12-hour light/dark cycle. To prevent cross contamination of uninfected mice and infected mice, cages were changed in uninfected or infected changing stations. Sentinel mice were tested quarterly for endo- and ectoparasites, mouse hepatitis virus, mouse parvovirus, and rotavirus, and annually for *Mycoplasma pulmonis*, pneumonia virus of mice, reovirus-3, Sendai virus, and Theiler's murine encephalomyelitis virus. Also, quarterly fecal colon samples were screened for *Citrobacter rodentium*, nonlactose fermenting *Escherichia coli*, *Salmonella* spp., *Klebsiella* spp., and *Clostridium* spp. (Phoenix Laboratories, Everett, WA). All ani-

mal procedures were approved by the University of Washington Animal Care and Use Committee.

Helicobacter Isolates

H. bilis was a natural isolate and kindly provided by L. Riley (University of Missouri, Columbia, MO) and *H. hepaticus* was obtained from the American Tissue Culture Collection (ATCC 51448; Rockville, MD). Organisms were streaked onto *Brucella* blood agar plates and grown under microaerobic conditions (90% N₂, 5% H₂, and 5% CO₂) in vented jars (Oxoid, Hampshire, UK) kept at 37°C. Bacteria were harvested and inoculated into flasks containing 150 ml of *Brucella* broth supplemented with 5% fetal bovine serum (Sigma Chemical Co., St. Louis, MO). The flasks were placed on a continuous shaker and incubated for 24 to 48 hours at 37°C in microaerophilic conditions. The organisms were centrifuged at 10,000 rpm at 4°C for 20 minutes. The resultant pellet was examined by gram stain and phase microscopy for purity, morphology, and motility. Organisms were confirmed to be catalase-, urease-, and oxidase-positive. The pellet was resuspended in *Brucella* broth and optical density was adjusted to 1.0 (OD₆₀₀) for an estimated 10⁸ CFU/ml.

Helicobacter Infection

Before infection, all mice were determined to be negative for *Helicobacter* spp. by fecal PCR.¹⁵ Mice were infected with 2 $\times$ 10⁷ CFU of *H. bilis* or *H. hepaticus* in a 0.2-ml volume by oral gavage two to four times.¹⁶ Study 1 comprised 90 *mdr1a*^{-/-} mice: *H. bilis* alone (n = 10), *H. hepaticus* alone (n = 20), *H. bilis* followed 5 days later by *H. hepaticus* (n = 20), *H. hepaticus* followed 5 days later by *H. bilis* (n = 20), or broth alone (n = 20). Study 2 used 40 *mdr1a*^{-/-} mice: *H. hepaticus* alone (n = 10), *H. bilis* followed 5 days later by *H. hepaticus* (n = 20), and broth alone (n = 10). Study 3 used 20 *mdr1a*^{-/-} mice: *H. bilis* followed 5 days later by *H. hepaticus* (n = 10), and broth alone (n = 10) and 20 FVB wild-type mice: *H. bilis* followed 5 days later by *H. hepaticus* (n = 10) and broth alone (n = 10). Confirmation of infection was done on pooled fecal cage samples taken at several points during the studies and at necropsy. Fecal samples were tested by PCR for cross-contamination with the other *Helicobacter* spp. in infected mice or for absence of *Helicobacter* infection in uninfected animals. Mice were weighed monthly and monitored weekly or bimonthly for weight loss, dehydration, and diarrhea. Mice were euthanized by CO₂ when they developed severe diarrhea or 20% body weight loss and samples were taken for histopathology, immunohistochemistry, and colonic epithelial cell preparations. Study 1 mice were euthanized at 15 to 40 weeks after infection at 4 to 10 months of age and study 2 mice were euthanized at 31 to 39 weeks after infection when $\sim$ 10 months old. Study 3 animals were euthanized at 23 weeks after infection and only used for Cox-2 immunohistochemistry.

Medicated Feed to Maintain Colitic Mice

In study 2, medicated wafers containing bismuth, amoxicillin, and metronidazole (Bio-Serve, Frenchtown, NJ) were fed to *mdr1a*^{-/-} mice to allow them to maintain body weight, decrease severity of disease, and favor the development of chronic colitis with dysplasia. To prolong survival of *mdr1a*^{-/-} mice with chronic colitis, all uninfected broth and dual-infected (*H. bilis* and *H. hepaticus*) mice were intermittently fed medicated wafers for a 2-month period at 6 to 16 weeks after infection and for a 1-month period at 18 to 20 weeks after infection. In study 3, all uninfected broth and dual-infected (*H. bilis* and *H. hepaticus*) *mdr* and FVB wild-type were given a medicated wafer regimen that started at 8 weeks after infection. The regimen included feeding medicated wafers for 1 week and regular rodent chow for 3 weeks. In both study 2 and study 3, diarrhea was ameliorated to some degree while animals were on medicated wafers.

Histopathological Analysis

At necropsy, animals were sacrificed by CO₂-asphyxiation. The gastrointestinal tract was isolated and the colon separated from the cecum. Samples of proximal colon and cecum were placed into 10% neutral buffered formalin or frozen in OCT-embedding material. Formaldehyde-fixed tissues were routinely processed and sections stained with hematoxylin and eosin. Cecum and proximal colon sections were graded by two blinded pathologists (H.B.O. and P.T.) for degree of IBD and dysplasia. Dysplasia was graded on a 1 to 4 scale (1, no dysplasia; 2, low grade; 3, high grade; 4, high grade with invasion/adenocarcinoma) incorporating criteria recently reported by Boivin and colleagues.²¹ Briefly, low-grade dysplasia was characterized by thickened mucosa with branching and elongated crypts with normal epithelial differentiation including goblet cells, maintenance of polarity, and nuclear morphology. High-grade dysplasia was characterized by thickened mucosa with elongate, irregularly branching glands, cytological and nuclear atypia including loss of differentiation and polarity, bunched and enlarged nuclei, and numerous mitotic figures.

Immunohistochemistry

MHC class II, β -catenin, CD44, presence of CD4⁺ T cells and macrophages (F4/80) were analyzed in colon tissue from four to six animals in each experimental group from study 2 animals. Cox-2 was analyzed from two animals from each group in study 3. Immunohistochemistry was performed on tissues snap-frozen in OCT or fixed in formalin using antigen retrieval. Briefly, 5- to 6- μ m frozen sections were fixed with acetone/ethanol (volume, 75%/25%) and 5- μ m paraffin sections were subjected to heat-induced epitope retrieval in 0.01 mol/L citrate buffer, pH 6.0, using a microwave oven for 10 minutes as described.²² Endogenous peroxidase activity was inhibited by sodium azide with trace amounts of nascent H₂O₂ produced by a glucose oxidase/glucose system.²³ Sec-

tions were incubated for 60 minutes with the following primary antibodies (M5/114, F4/80, RM4-5 for class II, macrophages, and CD4⁺ T cells, respectively; BD Biosciences, San Diego, CA; anti- β -catenin, Santa Cruz Biotechnology, Santa Cruz, CA; anti-Cox-2, Cayman Chemical, Ann Arbor, MI; anti-CD44, BD Biosciences, San Diego, CA). The secondary biotinylated antibodies were then applied for 30 minutes, followed by detection of signal with the avidin-biotin immunoperoxidase, Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). 3,3'-Diaminobenzidine was used as the chromogen. Negative controls included the exclusion of the primary antibody. All slides were evaluated by the pathologist blinded to experimental group.

Isolation of Colonic Epithelial Cells

Colonic epithelial preparations were done on four uninfected, four *H. hepaticus*-infected and seven *H. bilis*- and *H. hepaticus*-infected *mdr1a*^{-/-} animals from study 2 and two uninfected FVB+/+ mice. Colon was harvested and washed with Hanks' balanced salt solution (HBSS) without calcium, magnesium, or phenol red. Colonic epithelial cells were isolated following a minor modification of the method of Ogawa and colleagues.²⁴ Briefly, colon was minced to 3 to 4 mm in length and incubated in HBSS with 0.01 mol/L dithiothreitol for 10 minutes at room temperature. Then tissue pieces were placed in HBSS with 1.5 mmol/L ethylenediamine tetraacetic acid at room temperature for three 15-minute periods. At the end of each period, the tissues were agitated rapidly with a glass rod, the supernatants were aspirated, centrifuged at 1000 rpm 10 minutes at 4°C, and then the cell pellets were washed twice in HBSS and used for RNA isolation.

Cytokine Analysis by Multiplex Polymerase Chain Reaction

RNA was purified using a total RNA isolation kit TRI Reagent (Sigma, St. Louis, MO). IL-6, IL-1 α , IL-1R α , IL-1 β , and transforming growth factor- β 1 mRNA was analyzed by using Mouse Inflammatory Cytokine Set 3 Multiplex PCR kit (Biosource Int., Inc., Camarillo, CA). Interferon- γ , IL-10, IL-13, tumor necrosis factor- α , and IL-12p40 mRNA were also analyzed by using Mouse Cytokines CytoXpress Multiplex-PCR kit (Biosource Int., Inc.). PCR reaction tubes contained 5 μ L of cDNA and reagents provided by the kit. The following PCR profile was used for amplification: 95°C for 1 minute, followed by 6 cycles of 94°C for 1 minute and 60°C for 4 minutes, then 36 cycles of 94°C for 1 minute and 68°C for 2.5 minutes, and a final cycle of 70°C for 10 minutes. For Mouse Cytokines CytoXpress Multiplex-PCR kit, the following PCR profile was used for amplification: 5 cycles of 95°C for 1 minute and 60°C for 3 minutes, then 35 cycles of 94°C

for 1 minute and 65°C for 2 minutes. PCR product and 1× loading buffer were loaded onto 1% agarose gel and run at 100 V. The gel was stained with ethidium bromide and photographed.

RNA and cDNA Preparation and Real-Time Quantitative PCR

Ten μg of total RNA was treated with DNAase using Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, CA). DNA-free cDNA was generated by taking 2 μg of total DNase-treated RNA and adding 1 μl of 10 mmol/L dNTPs, 3 μl of random hexamers (stock 1:4 diluted in depC water; Invitrogen, Carlsbad, CA), and 6 μl of water. The tubes were heated at 68°C for 5 minutes, then cooled on ice for 1 minute. Four μl of first strand cDNA buffer (Life Technologies Inc., Grand Island, NY), 2 μl of 0.1 mol/L dithiothreitol, 1 μl RNase inhibitor (40 mg/ml, Life Technologies Inc.), and 1 μl of MMLV reverse transcriptase (RT) were added to each tube. A no RT control was also made for each reaction. The reactions were incubated at room temperature for 10 minutes, followed by 42°C for 1 hour, and heat inactivated at 70°C for 15 minutes. Two μl of the RT and no RT reactions were used for PCR with eIF1 α oligos to confirm the absence of DNA contamination. For real-time PCR, 1 μl of cDNA was amplified using SyBR green dye (Applied Biosystems, Foster City, CA) and PCR reactions were performed according to manufacturer's instructions (Brilliant SYBR Green QPCR Master Mix, Applied Biosystems). Reactions were amplified using ABI 7700 Prism thermocycler and sequence detector. All PCR reactions were run in parallel with β -actin as a loading control. No cDNA controls were also run with each sample to ensure purity. The efficiency of each primer set compared to β -actin was tested with serial dilutions of cDNA to ensure that they amplify equally. We then used the comparative C_T method to determine the amount of target cDNA according to the equation $2^{-\Delta\Delta C_T}$.²⁵ RT-PCR for *H. bilis* and *H. hepaticus* was done on fecal samples from animals in study 1 and study 2. Oligonucleotide sequences are as follows: *mdr1* 5' CGCTCCACTTATGATGCTGATCT; *mdr1* 3' TAGGCGTACGTG-GTCATTCTTCC; *IL1 α* 3'; GCAGGTCATTTAACCAAGTGTGC; *IL1 α* 3'; GATGAAGCTCGTCAGGCAGAAGTT; *IL1 α* 3'; AAGAAGGTGCTCATGTCCTCATCC; *IL1 α* 5'; CTCAG-GCAGGCAGTATCACTCAT; *p15* 5'; CCCTACCCAGTAA-GACAAAGCCAA; *p15* 3'; GGCCCGGGAAGTTCATCACTACT; *p21* 5'; AGTAGCAGTTGTACAAGGAGCCAG; *p21* 3'; CACCCACGGTATTCAACACTGACA; *c-myc* 5'; AC-CAACAGGAAGTATGACCTC; *c-myc* 3' AAGGCAGTAGC-GACCGCAAC; *H. hepaticus* and *H. bilis*.²⁶

Statistical Analysis

Bonferroni's multiple comparison test was used to detect significant differences at the $P < 0.05$ level between survival curves and degree of dysplasia.

Figure 1. Percent survival of *Helicobacter*-infected and uninfected *mdr1a*^{-/-} mice. The addition of *H. hepaticus* to *H. bilis* did not increase survival. *H. hepaticus*-infected *mdr1a*^{-/-} mice have significantly increased ($P < 0.01$) survival relative to all other groups.

Results

Co-Infection of *mdr1a*^{-/-} Mice with Both *H. bilis* and *H. hepaticus* Did Not Increase Percent Survival Compared to Infection with *H. hepaticus* Alone

Since we had shown that *H. bilis* induces severe inflammation and hyperplasia in *mdr1a*^{-/-} mice in a short period of time while *H. hepaticus* delays the development of colitis,¹⁶ we hypothesized that *H. hepaticus* infection might ameliorate (or delay) development of *H. bilis*-induced colitis and prolong survival. Therefore, the goal of study 1 was to compare colitis development and survival time in *mdr1a*^{-/-} mice that were singly infected (*H. bilis*) or dual-infected (*H. bilis*/*H. hepaticus*) to *H. hepaticus*-infected and to uninfected broth animals. Animals were terminated when they displayed diarrhea and 20% body weight loss. Survival curves for the various groups are shown in Figure 1. The addition of *H. hepaticus* to *H. bilis*-infected *mdr1a*^{-/-} mice having significantly longer ($P < 0.01$) survival relative to all other groups. The mean survival time was shortest in *H. bilis*-infected *mdr1a*^{-/-} mice (11.6 weeks) and the most prolonged in *H. hepaticus*-infected *mdr1a*^{-/-} mice (33.9 weeks) (Table 1). Survival time in broth *mdr1a*^{-/-} mice (16.9 weeks) was similar to *H. bilis*/*H. hepaticus*-infected (16.4 weeks) or *H. bilis*/*H. hepaticus*-infected (16.4 weeks) or *H. bilis*/*H. hepaticus*-infected (16.4 weeks) or *H. bilis*/*H. hepaticus*-infected (16.4 weeks).

Table 1. Survival Time (Weeks after Infection) in *mdr1a*^{-/-} Mice in Study 1

Infection	Mean survival time	Range of survival time	n
Broth	17.1 $\pm$ 6.4	7–32	20
<i>H. bilis</i>	11.6 $\pm$ 2.2	9–15	10
<i>H. hepaticus</i>	33.9 $\pm$ 8.4	10–41	20
<i>H. bilis</i> -> <i>H. hepaticus</i>	16.4 $\pm$ 8.3	7–32	20
<i>H. hepaticus</i> -> <i>H. bilis</i>	16.4 $\pm$ 8.5	8–40	20

Animals were necropsied when they had diarrhea and 20% body weight loss.

Figure 2. Incidence and degree of dysplasia in uninfected and *Helicobacter*-infected *mdr1a*^{-/-} mice (study 1). **A:** Infection of *mdr1a*^{-/-} mice with *H. bilis* alone or both *H. bilis* and *H. hepaticus* had the highest incidence of dysplasia. **B:** Dual-infected *mdr1a*^{-/-} mice exhibited the most severe dysplasia relative to uninfected mice ($P < 0.001$) or singly infected mice ($P < 0.05$); 1, no dysplasia; 2, low grade; 3, high grade; 4, high grade with invasion/adenocarcinoma).

cus-infected (16.7 weeks) *mdr1a*^{-/-} mice. Hence, addition of *H. hepaticus* to *H. bilis* did not prolong survival but rather contributed to development of colitis that was characterized by dysplasia as described below.

Colitis Was Characterized by Dysplasia More Frequently in *mdr1a*^{-/-} Mice Infected with *H. bilis* Alone or Dual-Infected with Both *H. bilis* and *H. hepaticus*

Although survival time differed among the groups, there were important histopathological differences in the degree of dysplasia. *Mdr1a*^{-/-} mice infected with *H. bilis* alone or dual-infected with *H. bilis* and *H. hepaticus* had the highest incidence of dysplasia (dysplasia score, >1; Figure 2A) but dual-infected *mdr1a*^{-/-} mice exhibited the most severe dysplasia relative to broth mice ($P < 0.001$) or singly infected mice ($P < 0.05$) (Figure 2B). Figure 3

Figure 3. H&E-stained paraffin-embedded sections of colons from *mdr1a*^{-/-} mice demonstrating the spectrum of mucosal lesions observed in study 1. **A:** Relatively normal mucosa from a nonclinically colitic *mdr1a*^{-/-} mouse. Note low-grade inflammation in the lamina propria confined to the mucosa. **B:** Hyperplastic colitis characterized by a diffuse moderately thickened mucosa with elongated glands, reduction in goblet cells, and increased mitotic figures. **C:** Low-grade dysplasia. Note irregularly branching glands, hyperchromatic nuclei, and loss of maturation gradient toward the lumen. **D:** High-grade dysplasia. Note irregular glands lined by crowded epithelial cells. The nuclei are pleomorphic, hyperchromatic, and stratified (inset). Original magnifications: $\times 10$; $\times 40$ (inset).

shows the spectrum of mucosal lesions noted. Hyperplastic mucosa was a feature seen in all *Helicobacter*-infected groups and was characterized by diffusely thickened mucosa with elongate glands, reduction in goblet cells, and increased mitotic figures (Figure 3B). Low-grade dysplasia was characterized by gland proliferation with branching, hyperchromasia of the epithelial cell nuclei, and loss of a cell maturation gradient toward the lumen (Figure 3C). High-grade dysplastic mucosa was characterized by thickened irregularly branching, elongated glands with nuclear atypia, loss of goblet cells, and epithelial polarity (Figure 3D). No evidence of invasive colonic carcinoma was seen in study 1 animals.

Alterations in MHC Class II, CD4, Macrophages, and Cox-2 in Colons from Uninfected and Helicobacter-Infected FVB Wild-Type and mdr1a^{-/-} Mice

Mdr1a^{-/-} mice showed increased expression of MHC class II in colonic epithelial cells independent of colitis or *Helicobacter* infection. Proinflammatory cytokines such as interferon- γ are increased in IBD^{27,28} and potentially responsible for enhanced expression of MHC class II in intestinal epithelial cells. As we previously reported in *Helicobacter*-infected IL-10^{-/-} mice,¹⁵ we noted increased expression of MHC class II in epithelial cells from proximal colon of *mdr1a^{-/-}* mice dual-infected with both *H. bilis* and *H. hepaticus* compared with proximal colon from FVB mice in which only lamina propria cells expressed MHC class II (Figure 4B). Notably, however, we also saw increased MHC class II expression in colonic tissues from uninfected *mdr1a^{-/-}* mice before there was minimal if any colonic inflammation (Figure 4F) suggesting these animals are primed to develop enteric disease. *Mdr1a^{-/-}* mice showed diffuse, very intense expression of MHC class II antigen in the enterocytes as well as lamina propria cells, which became more marked after *Helicobacter*-induced colitis and dysplasia (Figure 4J). Additionally, uninfected, noncolitic *mdr1a^{-/-}* mice had more CD4- and F4/80-positive cells in the proximal colon compared with uninfected FVB mice (Figure 4, G and H). The number of these cells increased in inflammatory infiltrates in dual-infected mice with colitis and dysplasia (Figure 4, K and L). Dual-infected *mdr1a^{-/-}* mice had large numbers of Cox-2-positive cells in the lamina propria of the proximal colon (Figure 5F) and cecum (data not shown). Uninfected *mdr1a^{-/-}* mice showed only rare faintly positive Cox-2-positive cells (Figure 5D, arrow) and dual-infected FVB wild-type mice had no Cox-2-positive cells (Figure 5B). No differences in intensity or distribution of β -catenin or CD44 expression were detected between dysplastic and nondysplastic crypts in individual animals, nor did there appear to be any differences in the expression of these two antigens between groups (data not shown).

Medicated Feed Was Used to Prolong Chronic Colitis and Contributed to the Development of High-Grade Dysplasia and Invasive Adenocarcinoma in Dual-Infected mdr1a^{-/-} Mice

The goal of study 2 and study 3 was to model chronic and relapsing IBD by allowing animals with chronic colitis to survive longer with the use of medicated wafers. Hence, in study 2, all uninfected and dual-infected mice were intermittently fed medicated wafers for a 2-month period at 6 to 16 weeks after infection and for a 1-month period at 18 to 20 weeks after infection. In study 3, all uninfected and dual-infected mice were intermittently fed medicated wafers (1 week on and 3 weeks off). Prolonged survival of uninfected and dual-infected *mdr1a^{-/-}* mice was achieved with the medicated wafers with a higher percentage (>50%) of dual-infected animals still alive by 39 weeks after infection (Figure 6) as compared with ~25% survival of dual-infected animals at 39 weeks after infection in study 1 (Figure 1). Nonetheless dual-infected *mdr1a^{-/-}* mice in study 2 had decreased survival ($P < 0.05$) relative to uninfected *mdr1a^{-/-}* mice and they also exhibited a high incidence of dysplasia (Figure 7A). However, more dual-infected *mdr1a^{-/-}* mice in study 2 exhibited a higher degree of dysplasia compared with uninfected *mdr1a^{-/-}* mice ($P < 0.01$) as shown in Figure 7B. Interestingly, singly infected *H. hepaticus mdr1a^{-/-}* had prolonged survival and a percentage of those animals also exhibited low- to high-grade dysplasia (Figure 7B). High-grade dysplastic mucosa was thickened by irregularly branching to cribriform glands with reduction in intraglandular stroma and goblet cells, increased nuclear and cytological atypia, loss of nuclear and epithelial polarity, and numerous mitoses. High-grade dysplastic lesions included two instances of medullary carcinoma *in situ* as recently described by Erdman and colleagues¹⁴ (Figure 8; D to F), and two instances of moderately differentiated adenocarcinoma with invasion of the muscularis mucosa by neoplastic glands (Figure 8; G to I).

Dual-Infected mdr1a^{-/-} Mice Have Increased Expression of IL-1 α / β in Colonic Epithelial Cells

We examined the expression of multiple cytokines between uninfected and *Helicobacter*-infected *mdr1a^{-/-}* mice and noted differences in the message for IL-1 α / β . There was increased expression of IL-1 α and IL-1 β mRNA in colonic epithelial cells from dual-infected *mdr1a^{-/-}* mice compared to uninfected *mdr1a^{-/-}* mice (Figure 9A). Changes in IL-1 α and IL-1 β expression were further quantitated by real-time PCR. First, we compared the expression of IL-1 α and IL-1 β mRNA in epithelial cell preparations from uninfected *mdr1a^{-/-}* mice compared to uninfected FVB control mice. We found that loss of *mdr1a* expression alone was sufficient to increase expression of IL-1 α , but not IL-1 β , an average of 300-fold when compared to FVB control mice. In contrast, expression of both IL-1 α and IL-1 β mRNA were increased an av-

Figure 4. OCT-embedded frozen colon section with immunohistochemical detection of MHC class II (**B, F, J**), CD4⁺ cells (**C, G, K**), and F4/80⁺ macrophages (**D, H, L**) in the colon of FVB wild-type (**B, C, D**), broth control (**F, G, H**), and dual-infected (**J, K, L**) *mdr1a*^{-/-} mice. In the normal, noninflamed colon of FVB mice (**A**), MHC class II expression is restricted to small numbers of leukocytes in the lamina propria (**B**) and numbers of CD4⁺ cells and macrophages are low (**C, D**). In contrast, there are subtle proliferative and inflammatory changes in broth-uninfected *mdr1a*^{-/-} mice (**E**), which is accompanied by pronounced up-regulation of MHC class II expression in enterocytes (**F**) and moderately increased numbers of CD4⁺ cells and macrophages in lamina propria (**G, H**). In animals dual-infected with *H. bilis* and *H. hepaticus*, there are severe proliferative and inflammatory changes in the colon (**I**), accompanied by significant up-regulation of MHC class II on enterocytes and infiltrating leukocytes (**J**) and marked infiltration of CD4⁺ T cells (**K**) and macrophages (**L**) in lamina propria. Original magnifications, $\times 10$.

erage of 600-fold in dual-*Helicobacter*-infected *mdr1a*^{-/-} mice relative to uninfected *mdr1a*^{-/-} mice (Figure 9C). These results suggest that loss of MDR1 expression is sufficient to result in increased local expression of IL-1 α , while infection with *Helicobacter* spp. are required for consistent elevation of both IL-1 α and IL-1 β mRNA levels. No differences were noted in the message for IL-6, transforming growth factor- β , interferon- γ , IL-10, IL-13, tumor necrosis factor- α , and IL-12p40 in epithelial cells from uninfected and *Helicobacter*-infected *mdr1a*^{-/-} and FVB mice (data not shown).

c-myc Is Increased in Colonic Epithelial Cells of Uninfected and Dual-Infected *mdr1a*^{-/-} Mice

To address how loss of MDR1 expression may result in dysplasia, we measured the expression of *c-myc*, an oncogene known to be deregulated in many cancers in humans, including colon cancer.²⁹ We found that loss of MDR1a expression alone was sufficient to result in a twofold to fourfold increase in *c-myc* expression in epithelial cell preparations from uninfected *mdr1a*^{-/-} versus FVB mice (Figure 9B). Infection of *mdr1a*^{-/-} mice with *H.*

Figure 5. OCT-embedded frozen colon sections from dual-infected FVB (**A** and **B**), uninfected *mdr1a*^{-/-} (**C** and **D**), and dual-infected *mdr1a*^{-/-} mice (**E** and **F**). **B**: Dual-infected FVB wild-type mice remain lesion-free and no Cox-2-positive cells are found in the colonic mucosa. **D**: Uninfected *mdr1a*^{-/-} mice show rare Cox-2-positive cells (**arrow**) in the mild, spontaneous inflammatory infiltrates of the colon. **F**: In contrast, dual-infected *mdr1a*^{-/-} mice have large numbers of cells in the lamina propria of colon (and cecum, data not shown) that express high levels of Cox-2. Note diffuse light background staining of intestinal contents, which is especially prominent in **B** and **D**. Original magnifications, $\times 20$.

bilis and *H. hepaticus* resulted in a further increase in *c-myc* expression to 5- to 12-fold (Figure 9C).

H. bilis Organisms Predominate in Dual-Infected *mdr1a*^{-/-} Mice

To understand how two species of *Helicobacter* could be interacting to induce colonic epithelial cell dysplasia, we used real-time PCR to quantitate *H. bilis* and *H. hepaticus* organisms in fecal samples of *Helicobacter*-infected mice (Figure 10).²⁶ In study 1, we found that oral gavage with *H. hepaticus* resulted in a significant infection at 20 weeks after infection, as measured by 100- to 1000-fold increased expression by real-time PCR over background (Figure 10A; Hh-1 and Hh-2). However, dual-infection of *mdr1a*^{-/-} mice with both *H. hepaticus* and *H. bilis* resulted

in apparent competition between *Helicobacter* spp. with overgrowth of *H. bilis* relative to *H. hepaticus*, as measured by the nearly complete obliteration of *H. hepaticus* expression in dual-infected animals (Figure 10A, Hb/Hh1-3). Interestingly, we found that animals infected with *H. bilis* alone still retained higher levels of *H. bilis* expression than dual-infected animals suggesting that *H. hepaticus* may somehow compete with *H. bilis* for nutrients or niches required to sustain maximal infection (Figure 10A, Hb1-3). Although the expression of *H. hepaticus* was below background in study 1, we were able to detect *H. hepaticus* in dual-infected animals in study 2 (Figure 10, B and C). In study 2, we found that medicated feed decreased *H. bilis* infection up to fourfold (Figure 10B) while medication decreased *H. hepaticus* up 16-fold or greater (Figure 10C). These results would suggest that medi-

Figure 6. Percent survival of *Helicobacter*-infected and uninfected *mdr1a*^{-/-} mice in study 2. Prolonged survival of uninfected and dual-infected (*H. bilis* and *H. hepaticus*) *mdr1a*^{-/-} mice was achieved by feeding medicated wafers. Dual-infected *mdr1a*^{-/-} mice had decreased survival ($P < 0.05$) relative to uninfected *mdr1a*^{-/-} mice and exhibited a high incidence of dysplasia.

cated wafers were effective in significantly reducing or eliminating some *Helicobacter* organisms allowing the animals to live longer due to a reduction in clinical gastrointestinal disease.

Figure 7. Incidence and degree of dysplasia in uninfected and *Helicobacter*-infected *mdr1a*^{-/-} mice (study 2). Dual-infected *mdr1a*^{-/-} mice had the highest incidence of dysplasia (A) and exhibited the most severe dysplasia relative to uninfected mice ($P < 0.001$) or singly infected mice ($P < 0.05$) (B). 1, no dysplasia; 2, low grade; 3, high grade; 4, high grade with invasion/adenocarcinoma.

Discussion

Human patients afflicted with IBD and chronic ulcerative colitis (UC) are at increased risk of developing colorectal cancer. The relative risk for colorectal cancer in UC patients is 10-fold greater compared to the general population.³⁰ Such patients are routinely screened for the degree of dysplasia with low-grade dysplasia associated with an ~10% risk of invasive carcinoma and high-grade dysplasia associated with an ~40% risk of carcinoma.³¹ Animal models used to study the transitions from hyperplasia to dysplasia, and dysplasia to neoplasia should ideally have phenotypic features that are comparable to the human disorders. An important risk factor for colorectal cancer in patients with IBD is the extent of colitis with increasing risk associated with more colonic surface involvement.³² *Helicobacter*-infected *mdr1a*^{-/-} mice develop chronic inflammatory disease that involves all of the cecum, and proximal, mid, and distal colon,¹⁶ and is characterized by hyperplasia with varying levels of dysplasia depending on the *Helicobacter* spp. This murine model may prove to be more useful to study the role of inflammation in colorectal cancer, since other reported models with intestinal polyposis³³⁻³⁵ develop more focal lesions with minimal inflammation. Also, the absence of p-glycoprotein in the intestinal epithelial cells of *mdr1a*^{-/-} mice³⁶ and its propensity for large bowel disease could have particular relevance for the study of gastrointestinal disease and cancer in humans. Although the MDR1 gene was first identified in multidrug-resistant tumor cells,³⁷ it is also expressed in intestinal epithelial cells^{38,39} although its physiological roles are unclear. There is accumulating evidence that p-glycoprotein is important in host-bacterial interactions in the gastrointestinal tract and maintenance of homeostasis.⁴⁰ Certain MDR1 gene polymorphisms have been suggested to confer susceptibility for ulcerative colitis¹⁸ and colorectal cancer^{20,41} and other epithelial tumors.¹⁹ In addition, a potential role of genetic polymorphisms of enzymes involved in metabolism of xenobiotics, is emerging as a risk factor in other tumors such as renal cell carcinoma.⁴² Studies in *mdr1a*^{-/-} mice suggest that the absence of the transporter alone is not sufficient to induce disease,¹⁶ but rather the presence of a particular bacterial trigger such as *Helicobacter* or members of the colonic flora are needed to initiate and sustain prolonged intestinal inflammation. Studies by Yamada and colleagues⁴³ and Mochida and colleagues⁴⁴ in *mdr1a*^{-/-}, *Apc*^{Min+} mice showed that the absence of p-glycoprotein suppressed intestinal polyp formation. However, this tumor model⁴³ differs from ours in that intestinal lesions do not show inflammation.

The relationship between chronic intestinal inflammation and the development of dysplasia and neoplasia is complex. In human patients with IBD, the degree of inflammation has been reported to correlate with dysplasia and cancer,⁴⁵ yet not all patients with chronic inflammation develop dysplasia or neoplasia suggesting the involvement of other factors. Based on experiments described here and previous reports by others, we propose that intestinal bacterial infections are one important pre-

Figure 8. Histopathology of high-grade dysplasia and invasive adenocarcinoma in dual-infected *mdr1a*^{-/-} mice. **A:** Colonic high-grade dysplasia (grade 3). **B:** Higher power of **A**. **C:** Cecal high-grade dysplasia (grade 3). Note haphazard stratification of hyperchromatic nuclei. **D:** Cecal high-grade dysplasia/medullary carcinoma *in situ*. **E:** Deeper serial section of **D**. **F:** Higher magnification of **D**. **G:** Invasive colonic adenocarcinoma. Note irregular glands present at the submucosa junction muscularis externa. **H:** Invasive colonic mucinous adenocarcinoma. **I:** Additional section of **H** demonstrating lake of mucin within a dilated submucosal lymphatic. Original magnifications: $\times 4$ (**A**, **G**); $\times 10$ (**B**, **D**, **E**); $\times 20$ (**F**, **H**, **I**); $\times 40$ (**C**).

disposing factor in the induction of colon cancer. Others have shown that bacterial infection with *Citrobacter rodentium* can promote colonic tumors in *Apc*^{Min+} mice in sites of bacterial-induced hyperplasia.⁴⁶ Our studies show that *mdr1a*^{-/-} mice infected with *Helicobacter* will eventually develop large bowel inflammation and dysplasia. In addition, we find that *Helicobacter*-induced dysplastic changes in colonic epithelial cells of *mdr1a*^{-/-} mice correlated with increased expression of the inflammation markers/mediators, MHC class II, Cox-2, CD4, F4/80, and the proinflammatory cytokines IL-1 β and IL-1 α . Importantly, we also find increased expression of the oncogene *c-myc*, a potent transforming gene in many human cancers. Human population-based studies suggest that the susceptibility to cancer increases when tissues are chronically inflamed, and the long-term use of nonsteroidal anti-inflammatory drugs reduces the risk of several cancers including colorectal cancer.⁴⁷ Here, we find that the absence of p-glycoprotein in the intestinal epithelial cell of uninfected *mdr1a*^{-/-} mice is *a priori* associated with a twofold to fourfold increase in *c-myc* and a 300-fold increase in IL-1 α . After dual infection of *mdr1a*^{-/-} mice with two *Helicobacter* spp., colonic crypts undergo dysplastic changes that are associated with a further increase (5- to 12-fold) in *c-myc* and a 600-fold increase in expression of IL-1 β and IL-1 α , and cyclo-

oxygenase-2 (Cox-2), all of which have been associated with dysplasia and cancer in humans. For example, *c-myc* is expressed in epithelial cells of patients with IBD⁴⁸ and increased expression has been reported in patients with IBD,⁴⁹ colon cancer,⁵⁰ and in Barrett's esophageal metaplasia and neoplasia.⁵¹ High *c-myc* protein expression correlates with *H. pylori*-associated gastric dysplasia and carcinoma,⁵² as well as with the degree of dysplasia and worsening prognosis in human patients with colorectal lesions.⁵³ *C-myc* expression has been correlated with increased apoptosis in colorectal cancer⁵⁴ and IL-1 protects transformed cells from apoptosis,⁵⁵ suggesting that IL-1 and *c-myc* collaborate during transformation. Consistent with this notion, certain IL-1 β alleles are associated with an increased risk for high-grade dysplasia or gastric cancer in human patients.⁵⁶⁻⁵⁸ Additionally, advanced dysplasia and cervical carcinoma in women is associated with increases in IL-1 β and IL-1 α ⁵⁹ and mucosal levels of IL-1 β increased before the development of cancer in a mouse model of *H. pylori*-induced gastric tumors.⁶⁰ The prostaglandin-synthesis enzyme Cox-2 is also highly associated with colorectal cancer and is expressed early in the development of colitis-associated tumors.⁶¹ Cox-2 expression is elevated in 85% of human colorectal cancers, and ~50% of colorectal adenomas^{62,63} and proposed mechanisms involve inhibition of

A

Figure 9. A: Multiplex PCR for multiple cytokines in colonic epithelial cells from uninfected and infected *mdr1a*^{-/-} mice. Increased expression of IL-1α and IL-1β was only noted in colonic epithelial cells of *mdr1a*^{-/-} mice infected with both *H. bilis* and *H. hepaticus* and not in epithelial cells from *H. hepaticus* or uninfected broth animals. Real-time PCR showing expression of *c-myc* and IL-1α and IL-1β in colonic epithelial cells from uninfected *mdr1a*^{+/+} and FVB^{+/+} mice (**B**) and from uninfected FVB^{+/+} and dual-infected *mdr1a*^{-/-} mice (**C**).

B

C

apoptosis and increased cell migration and angiogenesis in tumors. *C-myc* has been proposed to bind the *cox-2* promoter via association with the transcriptional regulators TIP49 and TATA-binding protein, resulting in increased expression of *Cox-2*.⁶⁴ Nonsteroidal anti-inflammatory agents that inhibit *Cox-1* and -2 reduce the size and number of colonic polyps in humans that harbor germ-line mutations in the tumor suppressor gene adenomatous polyposis coli (*APC*).⁶⁵

Although the relationship between p-glycoprotein, *c-myc*, *Cox-2*, and IL-1 are not proven in this animal model of colonic dysplasia and neoplasia, our data provide significant leads toward a mechanism of bacterial-induced colon carcinogenesis. From our studies, we propose a model whereby loss of MDR1 results in enhanced *c-myc* and IL-1 expression, which results in increased cell division and survival. Infection with *Helicobacter* results in further increases in expression of *c-myc*, IL-1, and *Cox-2*, which further increases the propensity for epithelial cells to divide and survive. Finally, increased inflammation associated with *Helicobacter* infection increases oxidative damage to cells, further increasing the chance for additional transforming events. In our *mdr1a*^{-/-} model, further studies using siRNA, anti-sense oligonucleotide, or gene targeting would be required to more formally test the roles of *c-myc*, IL-1, and *Cox-2* in modulating the development of epithelial cell dysplasia.

Helicobacter spp. may be a good prototype bacterial organism to study the mechanisms of bacterial-induced intestinal hyperplasia, dysplasia, and neoplasia and studies using both human and animal isolates have explored the pathogenesis. It is well documented that *H. pylori* is linked to gastritis, gastric adenocarcinoma, and malignant lymphoma in human patients.⁶⁶ Using HT29-19A intestinal epithelial cells, *H. pylori* has been shown to increase the passage of antigens across the epithelium and alone or in concert with IL-1β decrease epithelial cell integrity.⁶⁷ In animal models of gastritis and gastric lymphoma, *Helicobacter heilmannii* isolates have shown varying capacity for antigen-driven inflammation and associated disease.⁶⁸ *H. hepaticus* has been shown to induce hepatocellular carcinomas in susceptible strains of mice¹² that are not associated with mutations in p53 or Ras,^{69,70} and colorectal cancer has been induced in RAG2-deficient mice by infection with *H. hepaticus*.⁷¹ *H. bilis* has been associated with biliary tract malignancies in Japanese and Thai patients but a cause and effect has not been established.⁷² In this study, we found that the combination of infection with both *H. hepaticus* and *H. bilis* in *mdr1a*^{-/-} mice was most effective in producing dysplasia particularly if the chronic and relapsing disease process of human IBD was modeled with intermittent treatment with medicated wafers. This may be due in part to qualitative differences in the host immune response to

A

B

C

Figure 10. Real-time PCR for *H. bilis* and *H. hepaticus* from fecal samples from *mdr1a*^{-/-} mice. **A:** *H. bilis* is the predominant organism in dual-infected animals in study 1. **B:** Medicated feed has reduced numbers of *H. bilis* in dual-infected animals in study 2. **C:** Medicated feed has dramatically reduced *H. hepaticus* in dual-infected animals in study 2. Fold-expression was generated by the C^T method, whereby we compare the amount of *Helicobacter* DNA relative to β -actin DNA in the stool, and then comparing the normalized values to values generated in stool from broth-only animals.

these two *Helicobacter* species. We have previously shown that *H. bilis* induces severe inflammation and hyperplasia in *mdr1a*^{-/-} mice in a short period of time while *H. hepaticus* can delay the development of IBD in this mouse strain.¹⁶ The effect of *H. hepaticus* may be due to its ability to induce regulatory T cells (unpublished data)⁷³ and ameliorate the severe inflammation caused by the *H. bilis* thus allowing time for eventual transformation of epithelial cells. Additionally, other undefined bacterial species may compete with one another in the gastrointestinal tract and provide the necessary environment for progression to neoplastic lesions. Ongoing inflammation induced by certain bacterial species may result in oxidative stress-induced cell damage⁷⁴ increasing the frequency of genetic or epigenetic changes contributing to the promotion phase of carcinogenesis.⁷⁵ The *mdr1a*^{-/-} mouse with a propensity for IBD that can be modulated with *Helicobacter* spp. may be a useful murine model to determine critical factors involved in the progression of large bowel inflammation to colorectal cancer.

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