Colonic microbiota alters host susceptibility to infectious colitis by modulating inflammation, redox status, and ion transporter gene expression

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Ghosh S, Dai C, Brown K, Rajendiran E, Makarenko S, Baker J, Ma C, Halder S, Montero M, Ionescu VA, Klegeris A, Vallance BA, Gibson DL. Colonic microbiota alters host susceptibility to infectious colitis by modulating inflammation, redox status, and ion transporter gene expression. Am J Physiol Gastrointest Liver Physiol 301: G39–G49, 2011. First published March 31, 2011; doi:10.1152/ajpgi.00509.2010.—Individuals vary in their resistance to enteric infections. The role of the intestinal microbiota in altering susceptibility to enteric infection is relatively unknown. Previous studies have identified that C3H/HeOuJ mice suffer 100% mortality during Citrobacter rodentium-induced colitis, whereas C57BL/6 mice recover from infection. The basis for their differences in susceptibility is unclear and has been mainly attributed to differences in host genetics. This study investigated the role of the intestinal microbiota in altering susceptibility to C. rodentium-induced colitis. When the feces of C57BL/6 mice were gavaged into antibiotic treated C3H/HeOuJ mice, the C57BL/6 microflora led to a complete reversal in mortality patterns where 100% of the C3H/HeOuJ mice survived infection. This protection corresponded with reduced colonic pathology and less systemic pathogen load and was associated with increased inflammatory and redox responses with reduced epithelial cell death. C3H/HeOuJ mice are normally susceptible to infection-induced dehydration due to defective expression of colonic ion transporters such as Dra, CA IV, and CA I; expression of these genes was normalized when C3H/HeOuJ mice were colonized with the C57BL/6 microflora. Together, these data reveal that the microbiota is sufficient to overcome inherent genetic susceptibility patterns in C3H/HeOuJ mice that cause mortality during C. rodentium infection.

The intestinal microbiota plays a crucial role during the early development of both systemic and local immunity. In recent years, dysbiosis or altered bacterial communities within intestinal microflora have been implicated in a wide range of inflammatory diseases such as inflammatory bowel disease (IBD; 31), obesity (6), diabetes (23), celiac disease (7), and colonic neoplasia (47). However, it is still debated whether dysbiosis alters disease susceptibility or is a consequence of the disease itself. Hence, it is not known whether specific microbial community changes alter disease risk. It has been shown that the microbiota composition can confer colonization resistance toward pathogenic bacteria, and when the community is disturbed, like during infection and inflammation, this can lead to increased risks for enteric infection (26, 44). For the most part, the factors that alter the composition of the intestinal microbiota are elusive, although the presence of closely related species can increase the chance of invasion of a similar species into the gut ecosystem (42). The specific mechanisms of how microbiota may protect against enteric disease susceptibility are relatively undefined.

The aim of this study was to explore the potential role of the intestinal microbiota in controlling enteric disease susceptibility by using the resistant C57BL/6 and susceptible C3H/HeOuJ variation in susceptibility to infection and disease. As an example, studies in Brazil found that although most children exposed to EPEC suffered only acute infections, a smaller population of children suffered persistent infections that resulted in chronic diarrhea (10). Similarly, studies conducted with adult volunteers found that susceptibility to standardized EPEC inoculums varied substantially within a volunteer cohort (46). Furthermore, the severity of EHEC infection, including diarrhea and other clinical complications like hemolytic-uremic syndrome, has been shown to vary widely among infected individuals during EHEC outbreaks (21, 28, 33). Similar to humans, specific mouse strains demonstrate differential susceptibilities to C. rodentium, a natural murine A/E pathogen belonging to the same family as human-tropic EPEC and EHEC. C3H/HeOuJ mice are highly susceptible to C. rodentium infection and suffer 100% mortality in association with extensive tissue pathology, increased pathogen load (50), and severe dehydration caused by defective ion transport (5). In contrast, C57BL/6 mice develop a self-limiting acute colitis characterized by Th1 and Th17 responses with neutrophil and macrophage infiltration, crypt cell hyperplasia, mucodepletion, and barrier disruption with little associated mortality (4, 13–16, 22, 25). Although host-mediated defenses within the gastrointestinal tract impact an individual’s resistance to infections (8, 50), the potential for the intestinal microbiota to influence host susceptibility to infectious colitis has not been investigated.

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mouse strains infected with *C. rodentium*. We crossed microflora from C57BL/6 and C3H/HeOuJ mice and then examined survival and colonic responses during *C. rodentium* infection. We found that the intestinal microflora from C57BL/6 mice transferred protective responses when their stool was gavaged into the susceptible C3H/HeOuJ mice that were subsequently infected with *C. rodentium*. Although naive C3H/HeOuJ suffered 100% mortality during the course of infection, these mice when crossed with resistant C57BL/6 microflora were rescued and demonstrated 100% survival rates with reduced colonic damage and diminished systemic spread of *C. rodentium*.

Furthermore, survival of the C3H/HeOuJ mice crossed with C57BL/6 microflora was associated with increased gene expression of inflammatory cytokines TNF-α, IFN-γ, IL-22, and macrophage inflammatory protein (MIP)-2α, along with increased prooxidant responses known to limit *C. rodentium* infection. Finally, C3H/HeOuJ mice crossed with the microflora from C57BL/6 mice had elevated gene expression of the ion transporters Dra, carbonic anhydrase (CA) IV, and CA I; these genes are known to be critical for functional ion transport during *C. rodentium* infection (5). Although C57BL/6 mice had increased total microflora compared with C3H/HeOuJ mice, the protective responses of the microflora from C57BL/6 crossed into C3H/HeOuJ mice were associated with higher levels of bacteria from the phyla Bacteroidetes. These data indicate that resistant microbiota can protect mice from colonic damage and bacterial translocation following *C. rodentium* infection, overcoming the host’s genetic susceptibility.

**MATERIALS AND METHODS**

**Mice.** Six- to 8-wk-old C57BL/6 female mice (Charles River Laboratories breeders; Wilmington, MA) were bred in-house. Age-matched female C3H/HeOuJ mice were obtained from Jackson Laboratories (Bar Harbor, ME); all were housed individually in a temperature-controlled (22 ± 2°C) animal facility with a 12-h light-dark cycle. Mice were maintained under specific pathogen-free conditions and fed a standard sterile chow (Laboratory Rodent Diet 5001, Purina Mills, St. Louis, MO) as well as tap water ad libitum throughout the experiments. All procedures involving the care and handling of the mice were approved by the University of British Columbia Committee on Animal Care Ethics and under the guidelines of the Canadian Council on the Use of Laboratory Animals.

**Fecal crossing experiment.** Mice were randomly assigned to one of six groups: C3H/HeOuJ hosts receiving C57BL/6 microflora (S^R^ + R^M^); C57BL/6 hosts receiving C3H/HeOuJ microflora (R^H^ + S^M^); C57BL/6 hosts receiving C57BL/6 microflora (S^R^ + S^M^); C3H/HeOuJ hosts receiving C3H/HeOuJ microflora (S^R^ + S^M^); C3H/HeOuJ stool donor mice (S); and C57BL/6 stool donor mice (R). The microbiota in recipient postweaned 4-wk-old mice were depleted with antibiotics [ampicillin (1 g/l, Sigma), vancomycin (500 mg/l, Abbott Laboratories), neomyacin sulfate (1 g/l, Pharmacia/Upholhn), and metronidazole (1 g/l, Sidnak Laboratories)] provided in their drinking water for 4 wk. Three pellets (100 mg) of fresh stool from adult donor mice were collected and pooled in 1.2 ml sterile PBS; 100 μl of the mixture was gavaged into respective recipient mice four times over 12 days.

**Bacterial strains and oral infection of mice.** *C. rodentium* DBS100 were cultured overnight at 37°C in 5 ml of Luria broth (LB). Mice were inoculated by oral gavage using 0.1 ml of LB containing 2.5 × 10^5 colony forming units (CFU) of *C. rodentium*. Both C57BL/6 and C3H/HeOuJ mice were infected with the same bacterial preparation 3–5 days following fecal gavage in recipients.

Survival and body weight measurement. Mice were monitored for mortality and morbidity throughout infection and were euthanized if they showed signs of extreme distress. Survival data are presented as the percentage of the initial mice still surviving at each time point.

**Tissue collection and pathogen load.** Mice were euthanized at day 5–6 postinfection (pi) by cervical dislocation, followed by removal of the large intestine. Distal colonic and cecal segments were immersed in RNA-later (Qiagen) for real-time quantitative PCR analysis, flash frozen in liquid nitrogen and stored at −80°C for oxidation assays, or immersed in 10% neutral buffered formalin (Fisher) and processed for histological analyses and immunofluorescence. For pathogen counts, the spleen and mesenteric lymph nodes (MLN) were removed, weighed, homogenized in 1 ml PBS, serially diluted, and plated on McConkey agar plates, which were incubated for 24–48 h at 37°C.

**Microbiota analysis.** Similar to Lupp et al. (26), fresh stool was weighed and homogenized in 1 ml PBS, and 1:10 dilution of each homogenate was stored in 3% formalin at 4°C until use. For SYBR green DNA staining, samples were filtered onto Anodisc 25 filters (Whatman International) with a pore size of 0.2 μm and 2.5 cm diameter. After complete drying, each sample was stained with SYBR green I nucleic acid gel stain (Invitrogen). For fluorescent in situ hybridization (FISH), samples were filtered onto a polycarbonate membrane filter (Nucleopore Track-Etch Membrane, Whatman International) and dehydrated on the filter by soaking in 50, 80, and 100% ethanol. After air drying, the filter was incubated overnight at 37°C in the dark with hybridization solution (0.9 M NaCl, 0.1 M Tris pH 7.2, 30% formamide, 0.1% SDS) containing 250 ng each of the general eubacterial probe (EUB338: 5’–GCT GCC TCC CGT AGG AGT-3’), fluorescently labeled with far red, Bacteroidetes (Cytophaga-Flavobacter-Bacteroides) probe (CBB286: 5’–TCC TCT CAG AAC CCC TAC-3’) fluorescently labeled with Texas red, and γ-Proteobacteria probe (GAM42a: 5’–GCC TTC CCA CAT CGT TT-3’) fluorescently labeled with FITC. After incubation, the filters were washed in hybridization solution followed by wash buffer (0.9 M NaCl, 0.1 M Tris pH 7.2). Filters were dried and mounted on glass slides by use of ProLong Gold Antifade (Invitrogen) and viewed with a Zeiss Axio-Imager 2 microscope operating through Axioview software. Six to 10 fields per disc were randomly chosen and the number of cells was counted and averaged. Total microbiota load was calculated and percentages of Bacteroidetes and γ-Proteobacteria were calculated based on the number of positively probed eubacteria.

**Histopathological scoring.** To assess colonic pathology, we used a scoring system previously described (3) and adapted (13, 14). Paraffin-embedded colonic and cecal tissue sections from mice were stained with hematoxylin and eosin and examined by three blinded observers. For submucosal edema, submucosal leukocyte infiltration, goblet cell depletion, and crypt hyperplasia, 5–10 fields-of-view per section in each group were assessed at ×400 magnification. Infected groups were assigned a score relative to the control group average. Scores were calculated for submucosal edema (0, <100%; 1, 100–200%; 2, 200–400%; 3, 400–800%; 4, >800%), leukocyte infiltration (0, <100%; 1, 100–200%; 2, 200–400%; 3, 400–1,000%; 4, >1,000%), goblet cell depletion (0, >100%; 1, 51–100%; 2, 21–50%; 3, <20%), crypt hyperplasia (0, <100%; 1, 100–150%; 2, 150–200%; 3, >200%), and epithelial integrity (0, no change; 1, 1–10 epithelial cells shedding per crypt; 2, >10 epithelial cells shedding per crypt; 3, crypt ulceration; 4, crypt destruction). The scores of the controls were subtracted from their respective groups to obtain an accurate score of the pathological changes observed.

**Immunofluorescence and TUNEL staining.** Staining of tissues was performed as previously described (4), using primary antibodies that recognize myeloperoxidase (MPO, NeoMarker), F4/80 (Serotec), inducible nitric oxide synthase (iNOS, Upstate), and the translocated bacterial effector Tir (provided by Dr. W. Deng, Biotechnology Laboratory, University of British Columbia, Vancouver, BC, Canada) followed by secondary Alexa568- or Alexa488-conjugated goat anti-rabbit or anti-rat IgG antibodies (Molecular Probes) and ProLong...
Gold anti-fad reagent containing 4',6'-diamidino-2-phenylindole (DAPI; Invitrogen). The mean numbers of MPO + or F4/80 + cells were counted by a blinded observer under fluorescence in a ×200 magnification field of view. Cell death was measured by using the Trevigen TACS 2 TdT-Blue Label In Situ Apoptosis detection kit (Invitrogen). Tissue samples were deparaffinized, labeled, and mounted as per manufacturer’s instructions. Deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick-end labeling (TUNEL)-positive cells were counted by a blinded observer under bright-field illumination at ×200 magnification. The mean number of TUNEL-positive cells for each group was the total number of TUNEL-positive cells counted in 10 longitudinally sectioned crypts. Tissues were visualized via a Zeiss Axiosmager 2 microscope operating through Axiowiew software.

RNA extraction and real-time quantitative PCR. RNA was purified using RNaseasy kits (Qiagen). cDNA was synthesized with Superscript II Reverse Transcriptase and oligo(dT) 12–16mer (Invitrogen) followed by quantitative PCR on a MJ Mini-Opticon Real-Time PCR System (Bio-Rad) using IQ SYBR Green Supermix (Bio-Rad) and GAPDH, TNF-α, MIP-2α, IFN-γ, IL-1β, IL-22 (52), CA IV and CA I (32), and Dra (41). Quantification was determined by use of GeneExMacro OM 3.0 software (Bio-Rad) and is represented as the averaged fold expression relative to the uninfected control mice.

Western blotting. Following euthanization of mice, colonic tissues were homogenized and centrifuged, and supernatants were boiled in SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes by standard procedures. Membranes were incubated with anti-iNOS, superoxide dismutase (SOD)1, SOD2, and β-actin antibodies raised in rabbit (Santa Cruz Biotechnology), followed by secondary goat anti-rabbit horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology) and visualized by using an ECL detection kit (Amersham) with the Chemigenius detection system with use of GeneTools software (Syngene) for densitometric analysis. Values were expressed as a ratio to β-actin probed on the same blot as a loading control and denoted in arbitrary units.

Oxidized and reduced glutathione quantification. Colonic glutathione (GSH) content was measured by use of a commercially available kit (Trengren). Cecal tissue segments were homogenized and subjected to deproteinization with 5% metaphosphoric acid to remove protein thiol groups, snap frozen in liquid nitrogen, and stored at −80°C. GSH determination was performed within the next 48 h. GSH was reacted with 5,5'-dithiobis-2-nitrobenzoic acid to produce a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide GS-TNB produced is recycled back to GSH by glutathione reductase to produce more TNB. The rate of TNB production is directly proportional to this recycling reaction rate, which in turn is directly proportional to the total concentration of GSH in the sample. Oxidized glutathione (GSSG) was estimated by use of 4-vinylpyridine. The difference between total GSH and GSSG amounts gave the value of the reduced GSH. Protein assays were performed according to the Bradford method by using a nondeproteinated portion of the samples. Ratios are represented as infected values normalized to values obtained from uninfected control mice.

Statistical analysis. The results are expressed as means ± SE from three to eight mice per group per experiment. For comparisons of multiple groups, one-way analysis of variance with Tukey’s post hoc test was performed. For comparisons of two groups, Mann-Whitney U-test was performed. All analyses were performed by using GraphPad Prism 5 where P < 0.05 was considered significant.

RESULTS

Intestinal microflora from C57BL/6 mice protect against systemic bacterial disease. Since S mice are susceptible to increased systemic pathogen load (50), we examined whether S + R mice were protected from lethal colitis by decreasing the number of C. rodentium that could translocate across the epithelium and into systemic tissues. Mice infected with C. rodentium were euthanized and their spleens and MLN were removed for pathogen load by homogenizing tissues and plating on McConkey media for CFU. Results showed that S mice carried significantly higher pathogen levels in the MLN and spleen than R mice (Fig. 2A), similar to Vallance et al. (50). As expected, pathogen levels were high for both S + S and S mice and low for both R + R and R mice. Following infection, S + R mice demonstrated significantly reduced pathogen loads, corresponding with the reduced colonic damage scores (Figs. 1 and 2). In contrast, R + S mice carried...
significantly higher pathogen loads both systemically (Fig. 2A) and locally, as seen by specific antibody staining of *C. rodentium* in colonic tissue sections (Fig. 2B). These data show that S mice colonized with R microflora (*S*^H^+R^M^ or *S*^H^+S^M^) or colonized with each other’s microflora (*R*^H^+S^M^ or *S*^H^+R^M^) were infected with *C. rodentium* and assessed for lethal colitis. A: S mice crossed with R microbiota (*S*^H^+R^M^) survived infection similar to R mice unlike S infected mice, which suffered 100% mortality. The colons from the *S*^H^+R^M^ infected mice displayed less damage (B) microscopically (×200) with (C) significantly higher total damage scores than S mice carrying native microflora (S and S^H^+S^M^). Bars represent mean scores of damage assessed from colonic tissues from mice carrying microflora from R (■) or S (□) mice. Asterisks denote significantly greater damage in S and S^H^+S^M^ compared with S^H^+R^M^ or R and R^H^+R^M^; *p* < 0.05; 1-way ANOVA with Tukey’s post hoc test. p.i., Postinfection; ns, not significant.

**Intestinal microflora from C57BL/6 mice induce inflammatory cytokines.** We next addressed why S mice colonized with R microflora were protected from lethal colitis by examining host responses known to be important during *C. rodentium* infection (2, 4, 13–16, 22). Previous studies found that S mice were not dying of sepsis or the associated cytokine storm (5). Similarly, we found that S and S^H^+S^M^ infected mice had comparable expression of TNF-α, IFN-γ, and MIP-2α and significantly reduced IL-22 expression compared with R and R^H^+R^M^ infected mice (Fig. 3A). In contrast, S^H^+R^M^ mice infected with *C. rodentium* had a significant induction of IFN-γ, TNF-α, IL-22, and MIP-2α transcripts compared with S^H^+S^M^ infected mice (Fig. 3A). IFN-γ, TNF-α, and IL-22 are cytokines that have been previously implicated in host defense or *C. rodentium* killing (2, 17, 37, 38). This suggested that the R microflora induced cytokine expression important for reducing pathogen load in the large intestine of S mice.

Levels of monocyte chemotactic protein-1 were unchanged among all infected groups (data not shown); these observations were corroborated with quantification of F4/80-positive cells from immunofluorescent images revealing similar levels of macrophages infiltrating into infected colon tissues (Fig. 3B). Meanwhile MIP-2α, which is secreted by monocytes/macrophages and chemotactic for polymorphonuclear leukocytes, was significantly induced in the S^H^+R^M^ mice during infection. Correspondingly, quantification of MPO-positive cells from immunofluorescent images confirmed that significantly increased numbers of neutrophils infiltrated into the submucosa and mucosa of the colons in S^H^+R^M^ mice compared with S^H^+S^M^ mice during infection (Fig. 3B). Unlike the microflora from the
R host, the S microflora crossed into the R mice led to a significant reduction in TNF-α and MIP-2 gene expression during infection (Fig. 3B). This suggested that the loss of R microflora and/or the gain of S microflora inhibited cytokine gene expression in the colons of R mice. Overall, these results suggest that R microflora, when crossed into S mice, can induce inflammatory responses important for controlling C. rodentium infection.

Intestinal microflora from C57BL/6 mice induces oxidative responses protecting against epithelial cell apoptosis. C. rodentium induces iNOS expression in crypt epithelial cells in R mice, and the expression of this enzyme has been associated with oxidative stress and accelerated pathogen clearance (13, 49). To examine the possibility that R microflora can induce prooxidant responses we examined iNOS expression, antioxidant levels, and the ratio of oxidized/reduced glutathione (GSSG:GSH) in mice (Fig. 4, A and B). We found that R\textsuperscript{H}+R\textsuperscript{M} and S\textsuperscript{H}+R\textsuperscript{M} infected mice had upregulated iNOS protein expression, suggesting that the R microflora in either host induced this defensive response during C. rodentium infection (Fig. 4, A and B). The S microflora did not appear to induce a significant iNOS response in either host. Interestingly, the S\textsuperscript{H}+S\textsuperscript{M} infected mice had significantly higher expression of antioxidant MnSOD/SOD2 protein than S\textsuperscript{H}+R\textsuperscript{M} infected mice as well as R mice with either microflora (Fig. 4A). This suggested that the susceptibility of S\textsuperscript{H}+S\textsuperscript{M} mice to C. rodentium infection may reflect high endogenous antioxidant levels that reduce the oxidative stress response important for pathogen killing. This antioxidant response appeared to be downregulated by the R microflora since MnSOD was similarly decreased in both hosts carrying R microflora and unchanged in the presence of S microflora in the R host (Fig. 4A). We also examined the cytosolic antioxidant Cu/ZnSOD (SOD1), which was not dependent on microflora since S and R mice with...
Overall, the lack of oxidative stress in the large intestines of S mice display increased colonic epithelial apoptosis compared with R mice during C. rodentium infection (50). Whereas S mice had the highest epithelial cell death rate, the microflora from R mice was able to decrease cell death significantly in the colons of S mice during infection (Fig. 4C). We hypothesize that control of excessive epithelial cell death is important for the survival of $S^H + R^M$ infected mice since excessive epithelial cell death is associated with increased morbidity during C. rodentium infection (13–15, 50). The S microflora did not alter colonic epithelial cell death rates since $R^H + S^M$ mice were similarly low to $R^H + R^M$ mice during infection (Fig. 4C). Overall, these results demonstrate that R microflora induces prooxidant responses associated with decreased colonic epithelial cell death during infection.

Intestinal microflora from C57BL/6 mice induces ion transporter gene expression. Whereas this study and others have shown that S mice carry increased pathogen loads, both at localized and systemic sites (50), another important study revealed that S mice die from fatal dehydration due to defective expression of colonic ion transporters (CA IV, CA I, and Dra) during C. rodentium infection (5). The authors showed that S mice compared with R mice had impaired expression of CA IV, CA I, and Dra during infection and this resulted in decreased chloride absorption concurrent with significant water loss and the resulting mortality could be rescued with fluid treatment (5). Remarkably, we found that the microflora could modulate the expression of these genes. As seen in Fig. 5, S$H + S^M$ infected mice show significantly reduced expression of CA IV, CA I, and Dra genes relative to the R$H + R^M$ infected mice similar to the findings of Borenshtein et al. (5). In contrast, S$H + R^M$ infected mice had similarly increased gene expression of CA IV, CA I, and Dra compared with R$H + R^M$ infected mice (Fig. 5). Although we didn’t measure chloride absorption or water loss, we did note that S$H + R^M$ infected mice had stool similar to R, R$H + R^M$, and R$H + S^M$ infected mice in contrast to S and S$H + S^M$ infected mice, which often lacked solidly formed stools. Based on these observations and the findings of Borenshtein et al., our results suggest that the microflora from R mice can normalize CA IV, CA I, and Dra gene expression in the S mice to the level of R infected mice, which do not suffer from infection-induced fatal dehydration. R$H + R^M$ and R$H + S^M$ infected mice were similar in their expression levels of the transporter genes, indicating that the microflora from S mice did not inhibit CA IV, CA I, and Dra gene expression in R mice. Thus R microflora in S mice could rescue impaired gene expression of colonic ion transporters previously found critical for protection from excessive dehydration and associated mortality during C. rodentium infection.

Intestinal microflora from C3H/HeOuJ and C57BL/6 mice differ in composition and total bacterial loads. In the healthy mammalian colon, Bacteroidetes is one of the main bacterial phyla whereas Proteobacteria are relatively scarce (9, 24). To determine what differences in the intestinal microbiota from R and S mice could account for the protective host responses, we compared numbers of bacteria from the phyla Bacteroidetes and γ-Proteobacteria in stool samples of S and R mice. We used FISH analysis with probes specific for bacteria within Bacteroidetes and γ-Proteobacteria, as well as the eubacterial domain (all bacteria) to generate percentages for each group. We found that S mice had significantly fewer Bacteroidetes compared with R mice whereas γ-Proteobacteria were not significantly different (Fig. 6A). Additionally, we examined the
overall bacterial load using the nucleic acid stain SYBR green, since differences in bacterial load have been implicated in intestinal immune dysfunction (45). We found that S mice carried significantly lower total bacterial loads compared with R mice (Fig. 6A). Overall, these results indicate that the S mice have fewer Bacteroidetes in concert with an overall decreased bacterial load compared with R mice.

Considering that bacteria within Bacteroidetes have been shown to induce beneficial immune responses (48) and a reduced total bacterial load could mean differences in colonization resistance, we analyzed the microbiota from the fecal transplanted mice to determine whether these microflora differences between the S and R mice had been transferred (Fig. 6B). The results showed that significantly increased levels of Bacteroidetes were present in the stool of S^H+R^M compared with S^H+SM and similar to R^H+R^M mice. The R^H+SM had decreased levels of Bacteroidetes present in their stool compared with R^H+RM mice, but this was not significant. Although the total bacterial loads in stool from S^H+SM and R^H+R^M mice were significantly different, like S and R mice (Fig. 6, A and B), the stool from S^H+SM mice did not contain significantly more bacteria than S^H+SM mice. These results suggested that the increased levels of Bacteroidetes, not the total bacterial load, may be playing a role in the protective responses transferred from R microflora into the S mice during C. rodentium infection.

**DISCUSSION**

In this study, we examined the role that the intestinal microbiota plays in host susceptibility during C. rodentium infection in mice. Our results highlight the importance of the microflora as a prognostic factor that can modulate the severity of infection and the subsequent morbidity and mortality caused...
Tukey’s post hoc test. Reduced colonization and enterocolitis (43). Other than enteric expression.

Pathogen burdens, in association with increased proinflammatory survival corresponded with reduced tissue damage and lower microflora survived the acute colitis, similar to R mice. This found that when S mice were colonized with their own microflora they suffered mortality, similar to S mice, during infection. In contrast, 100% of the S mice colonized with R microflora had similar levels of bacteria. Thus it is more likely that the protective effects of the R microflora were conferred, at least in part, by increasing the numbers of Bacteroidetes in the S mice. Although this needs further clarification, Bacteroides sp. have been shown to be important for intestinal homeostasis and systemic immune responses (48). Additionally, we observed that R microflora increases colonic oxidative stress during infection, as measured by the increase in oxidized to reduced GSH ratio, induction of iNOS, and reduced antioxidant MnSOD/SOD2 protein expressions. In several models of inflammation, iNOS is responsible for production of large amounts of nitric oxide (NO). When antioxidants like MnSOD/SOD2 are low, excess free radicals including superoxide (O$_2^-$) either form hydrogen peroxide (H$_2$O$_2$) or combine with NO to form peroxynitrite (ONOO$^-$), which is extremely potent as an oxidant and has strong bactericidal activity against E. coli (20). Excess H$_2$O$_2$ produced during such prooxidant conditions also oxidizes available reduced GSH to its oxidized form (GSSG), thus altering cellular redox status. It is known that members of Bacteroides are oxygen tolerant and can survive oxidative stress (35). Although the Bacteroides-enriched microbiota in the R mice may be resistant to the induced oxidative stress by A/E bacteria. We transferred the microflora between resistant C57BL/6 and susceptible C3H/HeOuJ mice to examine the possibility that the intestinal microbiota plays a role in disease susceptibility to acute colitis caused by C. rodentium. We found that when S mice were colonized with their own microflora they suffered mortality, similar to S mice, during infection. In contrast, 100% of the S mice colonized with R microflora survived the acute colitis, similar to R mice. This survival corresponded with reduced tissue damage and lower pathogen burdens, in association with increased proinflammatory and oxidant responses and rescued ion transport gene expression.

Differences in microbiota composition have been recently reported to increase susceptibility to Salmonella enterica-induced colonization and enterocolitis (43). Other than enteric infection, the microbiota has been implicated in a number of other enteric diseases including IBD. The intestinal microbiota from IBD patients display decreased diversity (1, 29), with phyla-level reductions in Bacteroidetes and Firmicutes (11) and increases in Proteobacteria (18, 27, 36, 40). Additionally, the microflora from IBD patients has been shown to be unstable in both active disease and remission, in contrast to the microflora from healthy adults, which are relatively stable over time (36). Although microflora stability could ensure continued gut functions in healthy individuals, in other individuals whose intestinal communities contain fewer beneficial microbes or increased pathogenic microbes, the microbiota could be an initial factor that increases inherent disease risk. For example, dysbiotic microbiota has been shown to induce colitis in mice (12). Dysbiosis could contribute to enteric disease where specific populations of the microbiota have reduced protective features with subsequent alterations in immune responses (e.g., biased Th1 and Th17 responses and loss of immunological tolerance) or have more pathogenic properties promoting increased mucosal adherence/invasion activating immune cells in a detrimental manner (31). Although the mechanisms by which microbes regulating intestinal immunity are for the most part elusive, components of the microbiota are in constant communication with our innate immune receptors to maintain intestinal homeostasis (34), driving the expansion of B and T cells in Peyser’s patches and mesenteric lymph nodes, especially CD4+ T cells, including Foxp3-expressing T regulatory cells (19). Specifically, it has been shown that early colonization with Bacteroides fragilis can downregulate lipopolysaccharide responsiveness in infancy, revealing the importance of this particular species in inducing immune tolerance during early life (39).

Colonization with fewer beneficial microbes, like a reduction in Bacteroidetes, may influence enteric disease susceptibility through deregulated immune responses. In this study, we found that R mice carried more Bacteroidetes bacteria and a larger overall bacterial load compared with S mice. It is possible that the S mice were more susceptible to infection due to differences in total microbiota load; however, this is unlikely since after reconstitution both the $S^H+R^M$ and the $R^H+S^M$ had similar levels of bacteria. Thus it is more likely that the protective effects of the R microflora were conferred, at least in part, by increasing the numbers of Bacteroidetes in the S mice. Although this needs further clarification, Bacteroides sp. have been shown to be important for intestinal homeostasis and systemic immune responses (48). Additionally, we observed that R microflora increases colonic oxidative stress during infection, as measured by the increase in oxidized to reduced GSH ratio, induction of iNOS, and reduced antioxidant MnSOD/SOD2 protein expressions. In several models of inflammation, iNOS is responsible for production of large amounts of nitric oxide (NO). When antioxidants like MnSOD/SOD2 are low, excess free radicals including superoxide (O$_2^-$) either form hydrogen peroxide (H$_2$O$_2$) or combine with NO to form peroxynitrite (ONOO$^-$), which is extremely potent as an oxidant and has strong bactericidal activity against E. coli (20). Excess H$_2$O$_2$ produced during such prooxidant conditions also oxidizes available reduced GSH to its oxidized form (GSSG), thus altering cellular redox status. It is known that members of Bacteroides are oxygen tolerant and can survive oxidative stress (35). Although the Bacteroides-enriched microbiota in the R mice may be resistant to the induced oxidative stress...
responses in the colon during infection, these same responses specifically elicit cytotoxicity toward pathogenic C. rodentium, evident by reduced pathogen burdens in these mice. The reduced levels of C. rodentium in the presence of R microflora was associated with attenuated cell death in the intestinal epithelial cells, most likely because C. rodentium itself induces apoptosis and this has been shown to increase mucosal ulcerations and mortality (13–15, 50).

Among other pathways of protection, we also found that microflora from R mice induced a host-protective inflammatory response in the colon of S mice. Stool samples from C3H/HeOuJ (R) and C57BL/6 (S) mice (A) and R and S mice carrying microflora from R (solid bars) or S (open bars) mice were homogenized, filtered onto membranes, and hybridized with probes specific to eubacteria (all bacteria), Bacteroidetes, and γ-Proteobacteria for fluorescent in situ hybridizations analysis and were quantified by use of SYBR green nucleic acid dye. Membranes were examined via fluorescent microscopy to determine percentages of Bacteroidetes and γ-Proteobacteria from total bacteria or SYBR-positive cells from 6–10 random fields of view. Bars represent mean bacterial numbers. In A, asterisks denote significantly increased Bacteroidetes in R mice compared with S mice and significantly increased total fecal bacteria in R mice compared with S mice (*P < 0.05, Mann-Whitney t-tests). In B, asterisks denote significantly increased Bacteroidetes in R mice compared with S mice and significantly increased total fecal bacteria in R mice compared with S mice (dMM 10^13, 1-way ANOVA with Tukey’s post hoc test).

In summary, this study demonstrates that differences in host microbiota can dramatically alter host susceptibility to C. rodentium and colonization patterns in the gastrointestinal tract, which results in striking effects on the clinical course of infection. These results reveal that the microbiota and the host have a truly mutualistic interaction in the gut that can control those host responses that are beneficial and promote host survival during enteric infection. We are currently exploring
the cellular mechanisms underlying this susceptibility in mice since these finding could have important health implications if a similar form of susceptibility exists in humans. Not only is understanding the basis for host defense against A/E bacteria critical for identification of susceptible individuals and development of new therapies, but such findings may also shed light on how differences in individual host microflora contribute to the degree of intestinal protection to enteric infectious diseases. Finally, these data could have significant implications for our understanding of other enteric diseases that implicate the intestinal microbiota such as IBD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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