Development of fatal colitis in FVB mice infected with *Citrobacter rodentium*

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**ABSTRACT**

*Citrobacter rodentium* is the causative agent of transmissible murine colonic hyperplasia. The disease is characterized by severe but temporary epithelial hyperplasia with limited inflammation in the descending colon of adult mice on a variety of genetic backgrounds. The natural history of infection with this murine pathogen has been characterized in outbred Swiss Webster (SW) mice, but not in the cognate inbred FVB strain. In contrast to subclinical infection in SW mice, 12-week old FVB mice developed overt disease with significant weight loss and mortality beginning by 9 days post-inoculation (DPI). By 21 DPI, over 75% of infected FVB mice died or had to be euthanized, whereas no mortality developed in SW mice. Mortality in FVB mice was fully prevented by fluid therapy. Fecal shedding of bacteria was similar in both groups through 9 DPI, however a slight but significant delay in bacterial clearance was observed in FVB mice by 12-18 DPI. SW mice developed hyperplasia with minimal inflammation in the descending colon. FVB mice developed epithelial cell hyperproliferation, severe inflammation with erosions and ulcers, and epithelial atypia by 6 DPI in the descending colon. In the majority of surviving FVB mice, colonic lesions, including epithelial atypia, were reversible, although a small percentage (5-7%) exhibited chronic colitis through 7 months post-inoculation. The existence of susceptible and resistant lines of mice with similar genetic background will facilitate the identification of host factors responsible for outcome of infection and may lead to the development of novel strategies for preventing and treating infectious colitis.
INTRODUCTION

Diarrheagenic *Citrobacter rodentium* causes transmissible murine colonic hyperplasia (TMCH), a naturally occurring disease of laboratory mice characterized by epithelial cell hyperproliferation in the descending colon (reviewed in 33, 36). *C. rodentium* infection in adult mice of many outbred stocks and inbred strains is self-limiting, with little morbidity or mortality. In contrast, susceptible mice develop clinical signs of disease, including retarded growth, diarrhea, dehydration, coat ruffling, hunched posture, reluctance to move, recumbency, and high mortality. Susceptibility has been reported for suckling (2, 5, 6, 37, 38), helminth co-infected (14, 15), antibiotic pretreated (32), and adult mice of some inbred strains (7, 28, 31, 47, 56), as well as genetically engineered mice with varying types of immune defects (12, 35, 50, 56, 57, 59). The alterations in cell kinetics in TMCH are similar to those seen in idiopathic inflammatory bowel disease (IBD), including a 2- to 4-fold increase in labeling index and expansion of the proliferative zone (1). Furthermore, colonic mucosal hyperplasia associated with *C. rodentium* infection in mice serves as a promoter for colon tumorigenesis (3, 40), analogous to the increased risk for colorectal cancer seen in patients with IBD (8, 43, 44). Therefore, *C. rodentium* can be used as an animal model linking bacterial infection, IBD and colorectal cancer.

The importance of host genetic background in TMCH has been demonstrated, but not fully characterized. First reported by Barthold et al., (7), mouse strain susceptibility to *C. rodentium* infection was further investigated by Itoh et al. (29), and more recently by Vallance et al. (56). Differences in epithelial hyperplasia, mucosal inflammation, and mortality can be seen in different outbred stocks and inbred strains in response to bacterial challenge. Diet and indigenous microbiota also play important roles in the outcome of *C. rodentium* infection. Among different stocks and strains of mice, outbred Swiss Webster (SW) mice have been well
characterized as a model of TMCH (1-7, 17, 30, 32, 39, 46, 54, 55). FVB mice, widely used for creating transgenic animals, have been inbred from SW mice for homozygosity of the Fv-1\(^b\) allele that determines susceptibility to the B strain of Friend leukemia virus (52). Although FVB mice have been available since the late 1970s, there have been no reports of progression of TMCH in this inbred strain. The aim of this study was to characterize \textit{C. rodentium} infection in adult FVB mice and compare it to disease outcome in cognate outbred SW mice. Marked differences in host response to infection were observed in the two lines. While outbred SW mice developed subclinical disease with classic features of TMCH, FVB mice developed high mortality and severe colitis associated with epithelial atypia. This report characterizes \textit{C. rodentium} infection in FVB mice as a new model for studying fatal infectious colitis.
MATERIALS AND METHODS

Media, bacterial strains, and growth conditions. Lennox L broth (LB) and LB agar (Difco Laboratories, Detroit, Michigan) were used for routine cultivation of bacteria. MacConkey lactose agar (Difco) supplemented with 40 µg/ml kanamycin was used for bacterial detection in feces and tissues. Kanamycin resistant *C. rodentium* strain DBS120 (pCRP1::Tn5) (46), was used for infections.

Mouse infections. Specific pathogen free 12-week-old inbred FVB/NTac or outbred Tac:SW mice of both sexes (Taconic Laboratories, Germantown, N.Y.) were housed in polycarbonate microisolator cages within a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Animals were housed in a barrier facility and were viral antibody-free for 11 murine viruses and negative for enteric *Helicobacter* spp., *Salmonella* spp., and *Citrobacter rodentium*, as well as endoparasites and ectoparasites. The animals were maintained on pelleted rodent diet (LabDiet, Purina Mills, Inc., Richmond, Indiana) and water *ad libitum*. All experiments were approved by the MIT Animal Care and Use Committee. The experiments included 16 uninoculated control and 20 experimentally inoculated SW mice. The number of FVB mice in each experimental group, not including fluid intervention studies, are given in Table 1 and represent 5 independent experiments. Mice were inoculated by oral gavage with 100 µl of a bacterial culture grown overnight and concentrated 1:10 in LB broth (approximately 3-5 x 10⁹ CFU/mouse as determined by plate counts on MacConkey lactose agar) or with 100 µl of sterile LB broth. Mice were weighed prior to infection and body weight changes were calculated as a percent of initial body weight. Every three days, fecal shedding of *C. rodentium* was determined by plating serial dilutions of feces from individual animals on MacConkey lactose agar supplemented with kanamycin. The lower limit of detection was 1
CFU/mg of feces. In fluid intervention studies, infected mice received wet food from the day of inoculation and subcutaneous injections of 1 ml of Lactated Ringer's Solution (LRS, USP; Abbott Laboratories, Abbott Park, Illinois) twice a day beginning 6 DPI through 15-21 DPI, depending on body weight loss. In a pilot study, three groups of female FVB mice (uninoculated, infected without fluid therapy, and infected with fluid therapy; n = 5 per group) were followed for 21-28 days. In the chronic study, groups of uninoculated and infected female FVB mice with fluid therapy (n = 20 per group) were used. At 30 WPI, mice were euthanized and necropsied as described below.

As no gender differences were observed, the results for female and male mice were combined.

**Necropsy and histopathology.** Animals were euthanized with CO₂ at predetermined time points or when they lost ≥ 20% of body weight and/or exhibited severe clinical signs such as sunken eyes, hunched posture, reluctance to move, or recumbency. At necropsy, the entire colon and cecum of each mouse was collected aseptically and cleared of feces. The most distal 5 mm of the descending colon and the cecum were removed, homogenized in PBS and used for culturing bacteria by plating serial dilutions as described above. The rest of the tissues were snap-frozen in liquid nitrogen or fixed in neutral-buffered formalin for 24-48 hours, processed routinely, paraffin embedded, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). H&E-stained colonic sections were scored for pathological lesions by a veterinary pathologist (PRN) blinded to experimental groups. Inflammation within intestinal tissue sections was graded on a scale of 0 to 4 with ascending severity as previously described (20), where 0 corresponded to none, 1 to minimal, 2 to mild, 3 to moderate, and 4 to severe. The atypia noted among crypts was similarly graded. In chronic studies, the cumulative disease index was calculated as
(inflammation score x distribution score) + (hyperplasia score x distribution score) + (dysplasia score x distribution score). The maximum possible disease index was 48.

**Immunohistochemistry.** Immunohistochemical analyses of formalin-fixed paraffin-embedded intestinal sections from FVB mice were performed to assess bacterial attachment to epithelial cells, apoptosis and proliferation *in situ* as previously described (13, 20, 39). Polyclonal rabbit anti-*C. rodentium* (39) and anti–activated caspase 3 (Cell Signaling Technologies, Inc., Beverly, MA) antibodies, and monoclonal anti-BrdU antibodies (Dakopatts, Glostrup, Denmark) were used according to the recommendations of the manufacturer. Primary antibodies were detected with either biotinylated goat anti-rabbit IgG (Sigma) or using Animal Research Kit (DAKO, Carpinteria, CA). Sections were visualized using diaminobenzidine as substrate and counterstained with hematoxylin.

**Quantification of epithelial proliferation.** Animals received a single intraperitoneal injection of bromodeoxyuridine (BrdU; 50 mg/kg) from a freshly made stock solution of 5 mg/ml dissolved in PBS. The mice were euthanized 1 h later. The distal 4-5 cm of the colon were assessed for crypt length and for BrdU incorporation using quantitative computer-assisted image analysis (KS-400 MACROS). Only crypts visible along their entire length were analyzed. The number of specimen examined were n = 14 for control, n = 6 for 3 and 6 DPI, and n = 12 for 12 DPI colons with mean 21.6 crypts/mouse (n = 3-4 mice for cecum with mean 10 crypts/mouse). Crypt length was expressed in either cell number per crypt column or in μm. BrdU incorporation was expressed as the labeling index percent (LI%), which was calculated as a percentage ratio of labeled cells out of the total number of cells in the crypt. In addition, the proliferative zone was calculated as height of the highest positive cell to a total length of the crypt.
**Endpoint PCR for C. rodentium.** DNA isolated from frozen tissues using Qiagen DNeasy Tissue kit was amplified with primers specific for *eae* (ECW1, ECW2) as described (60). DNA isolated from DBS120 and *eae* mutant DBS255 (46) were used as positive and negative controls, respectively.

**Real-time quantitative RT-PCR.** Total RNA was prepared from frozen distal colon using Trizol reagent according to the recommendations of the manufacturer (Invitrogen, Carlsbad, CA). Five micrograms of total RNA were used to generate cDNA using SuperScriptII RT (Invitrogen) as recommended by manufacturer. Levels of IFN-γ, TNF-α, IL-10, and iNOS transcripts were quantified with Applied Biosystems predesigned primers and probes (TaqMan Gene Expression Assays) in an ABI Prism Sequence Detection System 7700 (Applied Biosystem, Branchburg, New Jersey, USA). Transcript levels were normalized to the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and expressed as fold change compared with averaged uninoculated FVB mice, using the Comparative Ct method (41).

**Statistics.** All data are presented as mean values ± the standard error of mean (SEM) unless otherwise indicated. Statistical analyses were performed with either GraphPad PRISM version 4.0 (GraphPad Software, Inc., San Diego, CA) or JMP 5.0.1 software (SAS Institute Inc., Cary, NC). The survival Kaplan-Meier curves were analyzed by log rank test and χ² analysis to determine median survival time. Comparisons between groups were made with a two-tailed Student *t* test, or paired *t* test (body weight loss). The differences in weight loss between infected and uninfected animals from both lines of mice were evaluated using two-way ANOVA test (for animal line and time post-inoculation) followed by Bonferroni post-tests. Statistical differences of colonic lesion scores between the groups were determined by using nonparametric Kruskal-
Wallis test followed by Dunn’s Multiple Comparison test. Gene expressions differences were analyzed by one-way ANOVA followed by Tukey’s Multiple Comparison Test. Whenever Bartlett’s test showed unequal variances, analysis of gene expression was performed on log 2 transformed data. A P value of < 0.05 was regarded as statistically significant.
RESULTS

FVB mice are highly susceptible to *C. rodentium* infection. As expected, *C. rodentium* infection in adult outbred SW mice failed to produce clinical signs of disease (data not shown). In contrast, 12-week old inbred FVB mice infected with the pathogen developed morbidity by 6 days post-inoculation (DPI), and mortality by 9 DPI with a median survival of 15 days (Fig. 1). By 21 DPI, only 23% of inoculated FVB mice survived compared to 100% survival in inoculated SW mice and in uninfected control animals (*P* < 0.0001 by log rank test). Mortality correlated with loss of body weight in infected animals (Fig. 2A). SW mice stopped gaining weight between 9 and 15 DPI (*P* < 0.05 by paired *t* test), while FVB mice lost weight beginning at 9 DPI (*P* < 0.0001 by paired *t* test). There was a recovery of body weight loss in FVB mice by 21 DPI, although only 23% of the inoculated animals were still alive at that time point, which may have contributed to the apparent increase. Body weight loss was significantly greater in FVB than in SW mice throughout the duration of the experiment (*P* < 0.0001 by two-way ANOVA compared with SW mice); at 9 DPI (5.3 ± 1.2% vs. 2.8 ± 1.4%), 12 DPI (17.5 ± 1.4% vs. 5.2 ± 2.1%), 15 DPI (20.2 ± 2.7% vs. 6.4 ± 2.3%), and 21 DPI (8.2 ± 1.6% vs. 6.0 ± 3.1%).

Bacterial load of *C. rodentium* in FVB mice. Fecal shedding of *C. rodentium* was detected beginning at 3 DPI, and the levels of fecal shedding were not significantly different between SW and FVB mice through 9 DPI (Fig. 2B). By 12 DPI, SW mice had reduced bacterial shedding and by 18 DPI the infection was cleared. Although FVB mice cleared the pathogen by 3 weeks post-inoculation (WPI), bacterial counts were higher than in SW mice by 12 DPI, indicating delayed clearance. Furthermore, since the most severely affected FVB mice often did not have any formed feces, the observed fecal counts may have underestimated the actual shedding of *C. rodentium* between 12 and 21 DPI.
Fecal shedding correlated with bacterial infection in the cecum and colon of FVB mice (Fig. 3A). Substantial attachment of *C. rodentium* to epithelial cells was observed in the colon at 6 DPI, with bacterial organisms on the luminal epithelial surface, as well as deeper in crypts (Fig. 3B panel c). The cecum, although comparable to the colon in level of infection at 6 DPI (Fig. 3A), did not exhibit *C. rodentium* attachment to epithelial cells (Fig. 3B panel d). By 12 DPI, *C. rodentium* organisms in the colon were mainly associated with exfoliated cells (Fig. 3B panel e), while attachment to epithelial cells in the cecum was occasionally observed (Fig. 3B panel f).

**Infected FVB mice develop marked colonic hyperplasia.** SW mice develop profound hyperplasia in the descending colon in response to *C. rodentium* infection (1, 7, 30, 54, 55). Infected FVB mice were found to have comparable hyperplasia characterized by increased colonic crypt height and changes in proliferation by 6 DPI (Fig. 4). By 12 DPI, the height of crypts doubled, and the proliferative zone expanded from 29.3 ± 1.2% to 84.3 ± 2.6% of the crypt column (*P* < 0.0001 by ANOVA). Mitotic figures and BrdU-positive cells were found on the surface epithelium (Fig. 4D), and the labeling index (LI) had increased from 8.9 ± 0.6% to 40.1 ± 2.4% (*P* < 0.0001 by ANOVA). Alterations in the cecum were less pronounced with a 1.5-fold increase in crypt length that was significantly different from uninfected control FVB mice (*P* < 0.01 by Student’s *t* test) and a tendency towards expanded proliferative zone and increased LI by 12 DPI that was not significantly different from uninfected control FVB mice (data not shown).

Apoptosis was analyzed by immunohistochemistry for activated caspase 3. Although occasional apoptotic cells were found in colonic crypts, the majority of activated caspase 3 was
associated with exfoliated epithelial cells in the lumen (data not shown), consistent with rapid removal of apoptotic cells from tissue.

**Infected FVB mice develop severe inflammation and associated epithelial atypia.** In agreement with published reports (6, 54, 55), SW mice developed limited inflammation in response to infection (data not shown). In contrast, infected FVB mice developed substantial inflammation, erosions and ulcers in the descending colon (Fig. 5A, \( P < 0.0001 \) using Kruskal-Wallis test with post hoc Dunn’s multiple comparison test compared with uninfected controls).

By 6 DPI, a minimal to mild mucosal infiltrate of granulocytes and mononuclear cells was present. By 12 DPI, infected FVB mice had multifocal coalescing moderate to severe mucosal inflammation with mucosal erosions, ulcers and submucosal edema (Fig. 5A, \( P < 0.001 \) compared with uninfected and 3 DPI mice, Fig. 6). Excessive mucus accompanied the inflammation in the proximal colon, resembling the catarrhal enterocolitis described by Brennan et al. (11). Lesions were characteristically most severe in the mid- to distal colon, milder at the cecocolic junction, and typically did not involve the proximal colon or the body of the cecum. Ileitis was rarely observed.

Inflammatory lesions were accompanied by dysplastic changes (Fig. 5B, \( P < 0.0001 \) using Kruskal-Wallis test with post hoc Dunn’s multiple comparison test compared with uninfected controls) that fit the definition of gastrointestinal intraepithelial neoplasia (GIN) from a recent consensus report (10). By 6 DPI, infected FVB mice had mild dysplasia in the descending colon, with crypt hyperplasia, minimal alteration of gland shape, and some loss of goblet cell differentiation, although moderate dysplasia was observed in a few animals at this time point (Fig. 5B, \( P < 0.01 \) and \( P < 0.05 \) compared with uninfected and 3 DPI mice respectively). By 12 DPI, a greater fraction of mice had moderate dysplasia (Fig. 5B, \( P < 0.001 \))
compared with uninfected and 3 DPI mice). The atypical glands were characterized by longer, hypercellular crypts with altered shapes (tortuous, branched), moderate loss of normal crypt orientation, and occasional cystic glands (Fig. 6D-F). Moderate cellular atypia was characterized by loss of goblet cell differentiation, plump and elongate nuclei with pseudostratification and nuclear hyperchromatism, loss of cell polarity, dark, elongate to oval, hyperchromatic and anisokaryotic nuclei with pseudostratification. Mice with the most advanced dysplastic changes had high nuclear-to-cytoplasm ratios, crypt branching with irregular buds, and occasional micronests within the abutting lamina propria (Fig. 6D). Approximately 65% of infected FVB mice had atypical crypts that had herniated into the GALT by 12 DPI (Fig. 6E and F). Dysplastic lesions were positively correlated with inflammation (Spearman r = 0.87, P < 0.0001). Similar changes, although less dramatic, were observed in the cecum by 12 DPI (data not shown), and here too dysplasia was positively correlated with inflammation (Spearman r = 0.729, P < 0.0001). These results indicate that FVB mice infected with *C. rodentium* may serve as a new model for studying infectious colitis and associated epithelial atypia.

**Mortality in infected FVB mice can be fully prevented by fluid therapy.** The cause of death in *C. rodentium*-infected mice is not clear. Undetectable serum TNF-α levels (below the limit of detection by ELISA of 15.6 pg/ml) and no or occasional minimal bacterial counts in draining lymph nodes or spleen at 9-12 DPI (data not shown) suggested that mortality was not due to systemic infection and septic shock. Moreover, signs of sunken eyes, ruffled coat, and decreased skin turgor in infected FVB mice were consistent with the clinical manifestations of dehydration (18). Indeed, the majority of moribund animals had severe diarrhea characterized by the lack of well-formed feces. To test the hypothesis that hypovolemia was the primary cause of fatality, fluid therapy intervention was employed. In a pilot study, daily subcutaneous administration of...
fluids from 6 DPI through 15-21 DPI was fully protective in infected FVB mice, whereas mortality was observed in infected mice that did not receive fluid therapy. Fluid therapy intervention did not affect bacterial shedding, alterations in body weight, or the severity or extent of colonic lesions in infected animals (data not shown). Moreover, mice in the intervention group still demonstrated diarrhea (lack of formed feces) and substantial body weight loss, but not signs of severe morbidity. These mice were active, had normal coat and eye appearance, and eventually recovered fully from disease. Successful fluid therapy allowed us to examine the long-term effects of acute *C. rodentium* infection in a longitudinal study.

**Chronic studies show reversibility of most of the lesions in FVB mice.** Morphologically, the features of regenerative atypia, including prominent crypt branching, occasional villous configuration of the surface, persistent mucus depletion and nuclear changes in the epithelial cells, can resemble GIN. This makes the histopathologic diagnosis more difficult (43). In order to assess possible progression of the lesions in infected FVB mice to neoplasia, the animals were followed for 16 and 30 WPI. In the first experiment, without fluid therapy intervention, only 15 out of 40 infected FVB mice survived. These animals were followed to 16 WPI. In a second experiment, fluid therapy was administered to 20 infected FVB mice, all of which survived, and the animals were followed through 30 WPI.

The lesions fully resolved in the majority of the mice (Fig. 7A), although the colonic disease score at 16 WPI was slightly, but significantly, greater than at 30 WPI or uninfected controls (P < 0.0001 by Kruskal-Wallis followed by Dunn’s multiple comparison test). This observation provides further evidence that the epithelial dysplasia was associated with active inflammation and was reversible. However, one of the mice in each of these two experiments (representing 6.7% and 5% for 15 and 20 post-infected mice at 16 and 30 WPI, respectively)
developed chronic lesions. A mouse at 16 WPI had moderate to marked inflammation and
hyperplasia with adjacent areas of normal mucosa that mainly involved the proximal colon and
cecum (Fig. 7B panel a). In a mouse at 30 WPI, similar lesions were found diffusely throughout
the large intestine and consisted of significant inflammation and hyperplasia, multiple lymphoid
aggregates throughout the mucosa, vascularized lamina propria, and rare crypt abscesses (Fig. 7
B panels b and c). At these time points the animals were no longer infected with \textit{C. rodentium}, as
judged by \textit{eae}-specific PCR (data not shown).

\textbf{Expression of pro-inflammatory and immunomodulatory genes in SW and FVB mice.}

Quantitative RT-PCR was performed on RNA isolated from full-thickness descending colon
tissue to evaluate the expression of some key genes involved in immune regulation in the
intestine, including the pro-inflammatory cytokines IFN-\(\gamma\) and TNF-\(\alpha\) and the
immunomodulatory cytokine IL-10 (Fig. 8). In addition, the expression of inducible nitric oxide
synthase (iNOS), which contributes to innate antimicrobial host defense by producing reactive
nitrogen species, was determined. The only difference found was in the expression of iNOS prior
to infection; uninoculated FVB mice demonstrated 7.5-fold lower expression of iNOS message
compared with control SW mice (\(P < 0.01\) respectively using one-way ANOVA followed by
Tukey’s multiple comparison tests). Infection with \textit{C. rodentium} induced expression of INF-\(\gamma\)
(12.5-fold, \(P < 0.05\)), TNF-\(\alpha\) (7-fold, \(P < 0.001\)), and iNOS (19-fold, \(P < 0.001\)) in SW mice
compared with uninoculated controls. IL-10 expression (1.6-fold increase) was not significantly
different than in uninfected SW mice. Similarly, infection in FVB mice stimulated expression of
proinflammatory genes; INF-\(\gamma\) (4-fold, not significant), TNF-\(\alpha\) (7-fold, \(P < 0.001\)), and iNOS
(79-fold, \(P < 0.001\)) compared with uninfected FVB mice. On the other hand, the expression of
IL-10 at 9 DPI had decreased 3-fold in infected FVB mice compared to uninfected controls (\(P <
By 9 DPI, the transcript levels of all genes were similar in FVB and SW mice.
DISCUSSION

A number of animal models have been developed to investigate different aspects of IBD, although none of them reproduce human disease completely. Models of intestinal inflammation can be either spontaneous or induced by administration of exogenous agents (chemicals or bacteria), gene targeting (knockout or transgenic rodents), and adoptive transfer of cells into immunodeficient animals (9, 26). Microbiota have profound effects on the host and particularly on the gut, including intestinal epithelium, the enteric nervous system, and mucosal immunity (19). Diarrheagenic *C. rodentium* is a unique murine pathogen that has been used to study important disease processes, including colitis. Infection is characterized by colonic hyperplasia with varying degrees of inflammation, depending on age, diet, microbiologic status and genetic background of the host. Here we report a new model of fatal infectious colitis in adult FVB mice infected with *C. rodentium*.

In contrast to subclinical infection in outbred SW mice, adult inbred FVB mice demonstrated a high degree of susceptibility. The 77% mortality was accompanied by profound inflammatory and erosive changes, including frank lower bowel ulcers, which correlated with morbidity. In contrast to susceptibility to infectious colitis, FVB mice are relatively resistant to DSS colitis, with milder lesions than ICR, C57BL/6, or BALB/c mice, and only 10% mortality 12 days after treatment with 5% DSS in the drinking water (25). Although they are immunocompetent, the degree of mortality seen in FVB mice infected with *C. rodentium* exceeds that reported in some immunocompromised mice. For example, little to no mortality following *C. rodentium* infection was reported in adult TNFRp55\(^{-/-}\), iNOS\(^{-/-}\) and IFN-\(\gamma\)\(^{-/-}\) mice (22, 23, 48) or in adult immunodeficient mice lacking immunoglobulin or T cells (34, 49). In addition, *C. rodentium* infection only caused mortality rates of 5% in adult Rag1\(^{-/-}\), 10-15% in
IL-12p40\textsuperscript{-/-} and 40% in mast cells-deficient animals (48, 49, 59). Importantly, adolescent mice between 3 and 5 weeks of age with these same immune defects are highly susceptible to \textit{C. rodentium} infection (12, 56, 57, 59). Furthermore, suckling inbred and outbred mice without such defects are also susceptible and exhibit a high degree of mortality (2, 5, 6, 37, 38). This suggests that susceptibility in young mice may be independent of innate or adaptive immunity, and may in fact be due to a failure to adequately compensate for fluid and/or electrolyte losses in the colon during \textit{C. rodentium} infection. Mice lacking functional T and B cells develop chronic \textit{C. rodentium} infection (34, 49, 57), demonstrating the importance of adaptive immunity in clearing the infection. Although infected FVB mice exhibited a relative delay in \textit{C. rodentium} clearance compared to SW mice, surviving mice and mice receiving fluid therapy intervention did successfully eliminate the infection by 3 WPI. Thus, any intrinsic immune defect that FVB mice have against \textit{C. rodentium} is partial and does not fully explain the susceptibility of this strain to disease.

To our knowledge, the only previously reported inbred strain of mouse with a consistently high mortality rate to \textit{C. rodentium} infection is the C3H strain. Different C3H substrains exhibit 50% to 100% mortality by 3 WPI independent of TLR4 status (3, 31, 56). C3H mice develop high levels of \textit{C. rodentium} in the colon as well as bacterial translocation to mesenteric lymph nodes, consistent with a defect in the clearance of infection. The mechanism of susceptibility of FVB mice to \textit{C. rodentium} infection appears to be different from that of C3H mice. For example, fecal shedding of \textit{C. rodentium} in FVB mice was similar to that seen in resistant SW mice. Furthermore, serum TNF-\alpha was not detectable by ELISA, and dissemination of bacteria to extraintestinal tissues was minimal and not associated with mortality (data not shown), allowing us to exclude bacteremia as the cause of death in infected FVB mice. Sick FVB
mice did exhibit signs of severe dehydration (18), including sunken eyes, reluctance to move, and decreased skin turgor. Based on clinical signs and the success of fluid therapy intervention, we suspect hypovolemia was the primary cause of fatality. The relative contribution of malabsorption and decreased fluid uptake, increased ion secretion, compromise of barrier function and/or exudation to \textit{C. rodentium} diarrhea, and the role of altered fluid balance in morbidity and mortality, remains to be determined.

The kinetics of \textit{C. rodentium} infection in FVB mice was comparable to that in Swiss Webster (39, 46, this report) and inbred strains of mice (56, 61). Similar to what has been reported in C57BL/6 mice as measured by bioluminescence imaging (61), FVB mice had 2 logs more \textit{C. rodentium} in the cecum than in the colon at 3 DPI. No \textit{C. rodentium} aggregates were detected in the colon of FVB mice at 3 DPI by IHC, also consistent with the lack of microcolonies made up of $\geq 10^3$ bacteria in the colon of C57BL/6 mice 3 DPI (61). By 6 DPI, extensive attachment of bacteria to the epithelial surface of the colon was found in FVB mice. By 12 DPI, the bacteria were no longer attached to the colonic epithelial surface, but were attached to exfoliated cells, which has been previously reported in Swiss Webster mice (30, 54, 55). \textit{C. rodentium} were abundant in the cecum of FVB mice by 6 DPI, but attachment to the cecal epithelial surface was not observed until 12 DPI. In contrast, Wiles et al. (61) reported attachment to the cecal lymphoid patch by 1 DPI in C57BL/6 mice that persisted focally without involving the remainder of the organ. The cecal patch is analogous to Peyer’s patches in the ileum and is present in the apical portion of cecum. Whether these differences are due to differing kinetics of infection in FVB versus C57BL/6 mice or to differences in IHC detection versus bioluminescence imaging of the cecal surface, following removal of cecal contents, remains to be determined. In any case, cecal lesions in FVB mice were associated with \textit{C.}
rodentium attachment and not simply the presence of organisms in the lumen of the organ (data not shown).

The increase in crypt column heights in the colon of FVB mice was comparable to that reported for SW mice (1, 7, 30, 54, 55), as well as in inbred strains (27, 40, 57, 58). Epithelial hyperplasia reflects an imbalance between proliferation and loss of cells arising in the crypt. We detected apoptosis by staining for activated caspase 3. Few apoptotic cells were identified in colonic crypt columns most likely due to their rapid removal from the mucosa. Strong activated caspase 3 signal was found primarily in extruded cells in the lumen of the colon. It remains to be determined if C. rodentium infection causes apoptosis in epithelial cells which are then shed into the lumen, or if infection and subsequent cytoskeletal rearrangements result in exfoliation of cells that then undergo anoikis, or perhaps both. In any case, some differences that have been reported with regard to the influence of C. rodentium infection on apoptosis may be explained by methodology. Evaluation of apoptosis in isolated crypts would exclude exfoliated cells, thus it is not surprising that no increase in colon apoptosis in SW mice was detected by TUNEL 12 DPI using this method (55). In addition, techniques that rely on DNA fragmentation such as the TUNEL assay are not specific for apoptosis, but also detect cells with DNA damage resulting from cell injury and other forms of cell death (24, 51). Thus, increases in TUNEL-positive cells in crypts in tissue sections from C3H mice (56) could correlate with cellular injury and DNA damage as well as apoptosis, which would be expected to exceed the number of apoptotic cells detected by activated caspase 3 staining.

Both FVB and SW mice developed comparable epithelial hyperplasia when infected with C. rodentium. FVB mice also developed severe mucosal inflammation associated with epithelial atypia (dysplasia). Inflammatory changes in the colon of FVB mice were similar to those in
mouse models of colitis that have been used to study idiopathic IBD (45). These lesions included erosions and frank colonic ulcers, infiltration of the lamina propria with neutrophils and mononuclear cells, crypt abscesses and gland atrophy, goblet cell depletion, epithelial hyperplasia, and epithelial atypia and dysplasia. Dysplasia has been identified as a precursor to neoplasia in IBD (43, 53), but there can be difficulties in distinguishing true dysplasia from regenerative atypia that can accompany severe inflammation (43). Consequently, chronic studies were carried out to determine the ultimate fate of these dysplastic changes. The majority of surviving mice in the absence of therapy or mice receiving fluid therapy intervention no longer had epithelial inflammation and dysplasia in the colon by 16 or 30 WPI, consistent with the complete resolution of lesions reported in SW mice (6, 30). However, a small fraction of animals (5-7%) developed chronic colitis. Persistent infection or recurrent infection could be excluded as a cause of these lesions by eae-specific PCR. There is also evidence for long-lasting or perhaps life-long immunity against rechallenge in convalescent animals following C. rodentium infection (1, 21, 34).

To test the hypothesis that cytokines could contribute to the dramatically different response of FVB and SW mice to C. rodentium infection, colon message levels of some key immunoregulatory cytokines were quantified. Despite initial differences in iNOS expression between uninoculated FVB and SW mice, infection as expected (22, 23, 27, 48, 57, 58) induced expression of proinflammatory genes to comparable levels in the two lines of mice. This suggests that neither IFN-γ nor iNOS or TNF-α play a critical role in determining the differential response of FVB and SW mice to C. rodentium infection. As reported in BALB/cByJ mice (16), IL-10 expression in SW mice slightly increased by 9 DPI, consistent with the induction of a regulatory T cell response (42) by C. rodentium infection. However, IL-10 expression in FVB
mice declined 3-fold by 9 DPI, suggesting a reduced capacity to regulate the inflammatory and immune responses directed against the pathogen in these animals. The differential IL-10 expression in the colon of FVB and SW mice could contribute to the distinct morbidity and mortality observed between these two cognate lines of mice. A protective role for IL-10 is in contrast to what has been reported for BALB/cByJ mice co-infected with a helminth *Heligmosomoides polygyrus* and *C. rodentium* (14, 15). This may reflect true differences between BALB/cByJ and FVB mice, or it may be that factors other than IL-10 contribute to the increased susceptibility to *C. rodentium* challenge during helminth infection. Studies to further define the role of immunoregulatory cytokines in susceptibility and resistance to *C. rodentium* infection are currently underway.

In a summary, we have developed and characterized a novel mouse model for studying fatal infectious colitis. FVB mice are immunocompetent, yet develop mortality similar to what has been reported in immunodeficient lines of mice that fail to control infection. To our knowledge, this is the first report demonstrating the protective effect of fluid therapy intervention. It remains to be determined if this approach will effectively prevent fatal colitis in immunodeficient lines of mice. The availability of susceptible and resistant lines of mice with very similar genetic backgrounds should facilitate the identification of host factors that determine the outcome of infection with *C. rodentium*. 
ACKNOWLEDGMENTS

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Table 1. Number of FVB mice used in experiments without fluid therapy intervention

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>3 DPI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>6 DPI</th>
<th>12 DPI</th>
<th>16 WPI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total</th>
</tr>
</thead>
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<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>Sterile broth</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>C. rodentium</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup>days post-inoculation, <sup>b</sup>weeks post-inoculation
FIG. 1. Experimental inoculation of FVB mice with *C. rodentium* causes significant mortality beginning by 9 days post-inoculation (DPI). By 3 weeks only 23% of infected animals remained alive. Survival curves were generated from 5 independent experiments (n = 41 for control and n = 84 for infected FVB mice). Differences between infected and control animals were significant (P < 0.0001 by the log rank test). There was no mortality in Swiss Webster mice with (n = 20) or without infection (n = 16).

FIG. 2. *C. rodentium* infection in FVB mice results in body weight loss and delayed bacterial clearance. (A) Body weight loss was greater in FVB mice than in SW mice beginning by 9 DPI (P < 0.0001 by two-way ANOVA). Shown are mean ± SEM of percent body weight change compared with initial body weight for surviving mice at each time point, which may overestimate the 21 DPI body weight change for FVB mice (see text). *P < 0.05 for SW and P < 0.0001 for FVB mice by the paired t test. (B) Infection kinetics were similar in both groups through 9 DPI, but bacterial clearance was delayed in FVB mice thereafter. Shown are mean ± SEM log 10 transformed CFU/g feces for surviving animals with feces at each time point, which may underestimate the fecal shedding of *C. rodentium* in FVB mice (see text). *P < 0.05 for 12 DPI and P < 0.0005 for 15 and 18 DPI by Student’s t test.

FIG. 3. Colon and cecum counts of *C. rodentium* do not correlate with bacterial attachment in FVB mice. (A) Tissue homogenates were plated at 3, 6, and 12 DPI. Shown are mean ± SEM log 10 transformed CFU *C. rodentium*/g tissue. (B) Although high *C. rodentium* counts were found...
at all time points in both the cecum and colon, there was little correlation with attachment of bacteria to epithelial cells identified by immunohistochemistry (IHC) for *C. rodentium* (original magnification 200x; panels a, c, and e are colon; b, d, and f are cecum). No *C. rodentium* was present in control colon or cecum of FVB mice (a and b). Bacterial attachment was observed in the distal colon beginning by 6 DPI. Bacteria are on the luminal surface, as well as deeper in the crypts (c). Although the lumen of the cecum contains bacteria, no attachment to epithelium was observed in the cecum at 6 DPI (d). By 12 DPI, most of the positive signal in the colon was associated with exfoliated cells (f), though some surface attachment was identified in more proximal regions of the colon (not shown). On the other hand, there were occasional areas of focal bacterial attachment to epithelial cells in the cecum at 12 DPI (f).

**FIG. 4.** *C. rodentium* infection causes epithelial hyperplasia in the colon of FVB mice. By 6 DPI, there was an increase in crypt column heights and labeling index (A) and expansion of the proliferative zone (B) in the distal colon of infected animals. Shown are mean ± SEM values, *P < 0.0001* by ANOVA with post-hoc Student’s *t* test comparisons between the groups. BrdU labeling in control (C) and 12-DPI colon (D) reveals extensive proliferation of enterocytes throughout the entire crypt including the surface epithelium in infected mice, original magnification 100x.

**FIG 5.** FVB mice infected with *C. rodentium* developed colonic inflammation by 6 DPI that was more pronounced by 12 DPI (A). This was accompanied by an increase in epithelial dysplasia (B) (*P < 0.0001* using Kruskal-Wallis non-parametric test with subsequent Dunn’s multiple comparison test). Each symbol represents one animal. Median lines are presented. Groups
indicated by different letters (a, b) are significantly different. Correlation analysis of inflammation versus dysplasia revealed a strong association between these two factors (Spearman r = 0.871, P < 0.0001).

FIG. 6. Microscopic lesions in the colon of FVB mice 12-15 DPI. (A) Normal colon from an uninoculated FVB mouse. (B) Hyperplastic crypts with increased numbers of mitotic figures and goblet cell depletion. Note significant infiltrate of inflammatory cells in the mucosa and submucosa. (C) Colitis and ulcer (arrow) with marked transmural inflammation and submucosal edema. (D) Epithelial atypia characterized by loss of normal tissue architecture, epithelial cell pleomorphism, gland malformation with splitting, branching and infolding. Some mice with more advanced lesions displayed high nuclear-to-cytoplasm ratio, crypt branching with irregular buds and occasional micronests within the abutting lamina propria. (E and F) GALT-associated atypia with herniated dysplastic crypts and dilated and attenuated cysts in submucosa. H&E stain. Original magnifications are 25x for panel E, 40x for C, 100x for A and F, 200x for B, and 400x for D.

FIG. 7. A small percentage of mice develop persistent lesions in the large intestine 16 or 30 WPI with C. rodentium. (A) Lesion scores in the colon and cecum of uninoculated mice and mice at 16 and 30 WPI. Each dot represents one animal. The colonic disease score was slightly, but significantly, greater at 16 WPI (P < 0.0001 by Kruskall-Wallis followed by Dunn’s multiple comparison test) compared with uninfected controls and 30 WPI. (B) Photomicrographs of chronic lesions. At 16 WPI, inflamed and hyperplastic tissue, mainly in the proximal colon was segmentally distributed (see adjacent normal mucosa). Original magnification 200x (a). At 30
WPI, diffuse lesions were observed throughout the colon and cecum. Note the numerous lymphoid aggregates, crypt abscesses and dilation. Original magnification 100x (b and c).

FIG. 8. Quantitative RT-PCR for expression of immunomodulatory cytokine genes. Susceptible FVB and resistant SW mice demonstrated comparable expression of proinflammatory and immunomodulatory genes in response to *C. rodentium* 9 days post-inoculation. Each symbol represents one animal. Mean lines are presented.