A Model of *Salmonella* Colitis with Features of Diarrhea in *SLC11A1* Wild-Type Mice

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Abstract

**Background:** Mice do not get diarrhea when orally infected with *S. enterica*, but pre-treatment with oral aminoglycosides makes them susceptible to *Salmonella* colitis. However, genetically susceptible *ItyS* mice (Nramp1<sup>169D</sup> allele) die from systemic infection before they develop diarrhea, so a new model is needed to study the pathogenesis of diarrhea. We pretreated *ItyR* mice (Nramp1<sup>169G</sup>) with oral kanamycin prior to infecting them with virulent *S. Typhimurium* strain 14028s in order to study *Salmonella*-induced diarrhea. We used both a visual scoring system and the measurement of fecal water content to measure diarrhea. BALB/c.D2<Nramp1> congenic started losing weight 5 days post-infection and they began to die from colitis 10–14 days after infection. A SPI-1 (*invA*) mutant caused cecal, but not colonic inflammation and did not cause diarrhea. A *phoP*<sup>−</sup> mutant did not cause manifestations of diarrhea in either normal or NADPH-deficient (*gp91<sup>phox</sup>*) mice. However, strain 14028s caused severe colitis and diarrhea in *gp91<sup>phox</sup>-deficient* mice on an *ItyR* background. *pmrA* and *F* mutants, which are less virulent in orally infected BALB/c mice, were fully virulent in this model of colitis.

**Conclusions:** *S. enterica* must be able to invade the colonic epithelium and to persist in the colon in order to cause colitis with manifestations of diarrhea. The NADPH oxidase is not required for diarrhea in *Salmonella* colitis. Furthermore, a *Salmonella* *phoP* mutant can be cleared from the colon by non-oxidative host defenses.

Introduction

*Salmonella* gastroenteritis is a global health problem, even in industrialized nations. The Center for Disease Control (CDC) estimates that there are nearly 1.4 million foodborne *Salmonella* infections annually in the USA [1]. Even though most *Salmonella* intestinal infections are self-limited, the illness usually lasts 3–5 days and is estimated to cost $1.4 billion/year in lost wages, expense of recalls, and medical costs [2]. Furthermore, there has been a steady increase in the prevalence of antibiotic resistant *S. enterica* serovar *Typhimurium* (hereafter called *S. Typhimurium*) [3], and a recent study from CDC found that 20% of victims in food borne epidemics caused by antibiotic resistant *S. enterica* required hospitalization, emphasizing that even gastroenteritis can be a serious disease [4].

*S. enterica* are invasive enteric pathogens, but the pathogenesis of diarrhea in *Salmonella* gastroenteritis is poorly understood. Many investigators have focused on mechanisms of cell invasion using cultured epithelial cells. This led to the discovery of a type 3 secretion system (TTSS) that is encoded by a *Salmonella* pathogenicity island (SPI-1). The TTSS mediates epithelial cell invasion by injecting bacterial products into host cells that rearrange the host cytoskeleton, causing localized membrane ruffling where the bacteria enter [5],[6],[7]. Uptake of *Salmonella* also triggers a complex transcriptional response in epithelial cells that results in expression of many NF-κB regulated genes. Since most of those genes encode pro-inflammatory cytokines and chemokines this response has the potential to initiate inflammation, and inflammation is a prominent feature of *Salmonella* gastroenteritis. The induction and basal secretion of chemokines by polarized epithelial cells occurs rapidly [8], [9]. Many of the *Salmonella*-induced changes initially observed in cultured cell lines have been confirmed in vivo in bovines and in human intestinal xenografts in SCID mice [10],[11]. However, it is likely that *Salmonella* induce additional epithelial responses that are not apparent in vitro because cell lines do not recapitulate all of the properties of the native intestinal epithelium.

Mice do not develop diarrhea when orally infected with *S. enterica*, which has greatly hampered the study of the pathogenesis of intestinal infection. However, a mouse model of *Salmonella* colitis in streptomycin-pretreated mice was developed more than fifty years ago by Bohnhoff [12]. Over the subsequent 20 years several groups used this model to show that oral streptomycin makes mice 100,000 fold more susceptible to oral *Salmonella* infection, and that alterations in the normal intestinal microflora are responsible for their increased susceptibility. Indeed, germ free mice are also
susceptible to *Salmonella* colitis [13]. This model languished until Harth’s group in Switzerland recently revived it. They confirmed that the streptomycin-treated BALB/c and C57BL/6 (B6) mice get an acute colitis when infected orally with *S. Typhimurium* [14]. Most importantly, they showed that SPI-1 mutants of *S. Typhimurium* (invG) did not produce colitis, though they still could infect the cecum as well as wild type (WT) *Salmonella*, and they were also invasive as measured by CFU in the mesenteric lymph nodes [14]. Hapfelmeier et al [15] then showed that SPI-1 effector molecules SipA, SopE, and SopE2 were involved in producing the pathology of *Salmonella* colitis. Thus, they established the physiological relevance of the model.

Nearly all reported experiments on *Salmonella* colitis in mice have been done with *Nramp1* mutant mice (i.e., S. typhimurium, Stillburg) that are so susceptible to systemic *Salmonella* infections that they die 4–5 days after oral infection. Perhaps because of that, most studies of *Salmonella* colitis in mice have focused on cecal inflammation [15], [14], [16]. Although investigators refer to it as a model of *Salmonella* colitis, inflammation and gross pathology are focused on the cecum with some inflammation in the contiguous colon. *Nramp1* is encoded by a gene on chromosome 1 that is now called *Slc11a1* [17]. Normal animals do not have mutations in this gene. We began to study *Salmonella* colitis in *ItyR* (Nramp1) mice. This paper reports our results with this model of colitis, a model of pan colitis with features of diarrhea.

**Results**

We first compared the natural history of oral infection with *S. Typhimurium* in kanamycin-treated BALB/c and BALB/c *D2* *Nramp1* mice. As expected, all the BALB/c mice were dead by 5 days after infection and they had 10^6–10^7 CFU/spleen at the time of death. We expected the BALB/c *D2* *Nramp1* congenic mice to survive and to spontaneously clear the infection, but instead they started to lose weight by day 5–6 after infection (Figure 1A) and they had visible evidence of diarrhea as manifested by fecal soiling of their peri-anal fur. By day 14 post infection the mice had lost about 30% of their body weight. We repeated the experiment to do quantitative microbiology on the tissues (Figure 1B). Between days 2 and 8 after infection there was a 1 log increase in the number of *Salmonella* in the mesenteric nodes and a 3 log increase in the number of bacteria in the spleen, but over the next five days there was no further increase in numbers of Salmonella in those organs, even though about half the remaining mice in this experiment died between days 8 and 14 after infection. Numbers of gentamicin-resistant (intracellular) *Salmonella* in their ceca remained fairly constant throughout this time period. Even though BALB/c *D2* *Nramp1* congenic mice could not clear the intestinal infection, colony counts of *Salmonella* in the spleen were well below the numbers that were found in genetically susceptible BALB/c mice at the time of death. On day 2 after infection inflammation was limited to the cecum (not shown), but by day 8 after infection in the pathologic process had progressed to involve the entire colon with many mucosal ulcers scattered throughout (Figure 2), and the mice had watery feces in their distal colon, a feature of diarrhea.

To investigate the role of SPI-1 in the pathogenesis of colitis and diarrhea we infected BALB/c *D2* *Nramp1* mice with an invA mutant of 14028s, which has no functional SPI-1 secretion system and so cannot invade epithelial cells in vitro [18]. Mice infected with this mutant lost less than 10% of their body weight and by day 8 after infection they had a mean diarrhea score of only 2, compared to a diarrhea score of >4 in mice infected with WT (Figure 3). Furthermore, the mean water content of the feces on day 8 was no greater than that of uninfected control mice, while feces from WT infected mice were nearly 80% water (Figure 3B). As Barthel et al previously reported, after 48 hours there are nearly equal numbers of SPI-1 mutants and WT *Salmonella* in the feces, cecal wall, and mesenteric nodes (data not shown). However, by day 8 after infection there were approximately 10 times more WT bacteria than invA mutants in ceca, and the differences were even greater in the feces, the mid and distal portions of the colon (Figure 3C). There also were more WT than invA organisms in the mesenteric nodes and spleen, though the differences between the groups were not as great as we found in the colon samples and they may have reflected the difference in the extent of infection in the colon. Cecal inflammation remained prominent in the invA group on day 8 post-infection (Figure 4A) but there was essentially no inflammation in the mid or distal colon (Figure 4).

We then infected BALB/c *D2* *Nramp1* mice with a *phoP* mutant [19]. On day 2 after infection *phoP*-infected mice had a mean diarrhea score of 2.5 and an increased water content of their feces, both of which were similar to results from WT infection (Figure 5A&B). However, by day 8/9 after infection the *phoP* diarrhea score had decreased to 1.5 and the water content of their colonic feces was now normal, whereas the water content of colonic feces from mice infected with WT *Salmonella* was ~80%. On day 8 after infection there were significantly fewer *phoP* than WT *Salmonella* in the stool, cecum, and mesenteric nodes (Figure 5C). The pathology in the cecum also changed between day 2 and 9 after infection: on the second day the pathology was indistinguishable from a WT infection (Figure 6A), but on day 9 the mucosa looked essentially normal (Figure 6B). The colons were...
We then turned our attention to investigating host factors that might contribute to diarrhea. *Salmonella* gastroenteritis is an inflammatory condition with large numbers of PMN in the tissues for at least the first 5 days after infection [15],[14]. Because neutrophils can make a vigorous oxidative burst and people with CGD are more susceptible to *S. enterica* bacteremia [20], we tested the role of reactive oxygen in both host defense and in mediating diarrhea. We infected mice with chronic granulomatous disease (CGD) due to a targeted mutation in *ggd* on the X chromosome [21]. We crossed homozygous *ggd* mutant females with transgenic TF6 males so that male offspring had CGD and female offspring were carriers. Both sexes inherited one allele of the *Nramp1* transgene, which is dominant, so they were IyR. After 48 hours both male and female mice had inflamed ceca (not shown) and there was no significant difference between their diarrhea scores or in the numbers of *Salmonella* that we recovered from their ceca (Figure 8). We terminated the experiment on day 7 because 1/2 of the infected males with CGD had died (none of the female carriers died). However, all the female carriers of the *ggd* mutation had full blown diarrhea by day 7 (mean diarrhea score 4.5) as happens in IyR mice, and the 3 remaining males with CGD all had diarrhea scores of 5 (Figure 8A). As expected, we recovered significantly more *Salmonella* from the male CGD mice than from the female carriers on day 7 (Figure 8B).

Since it has been reported that *phoP* mutants are more susceptible to oxidative killing in vitro [22], we asked whether *phoP* mutants would be more virulent in CGD mice, and they were not. Neither the males nor the females infected with the *phoP* mutant had diarrhea on day 8 after infection (Figure 9A). As we had seen with BALB/c.D2 *Nramp1* congenic mice, the *phoP* mutant bacteria were able to invade the intestinal wall, but by day 8 they were nearly completely eliminated from the ceca and mesenteric nodes of both CGD and control mice (Figure 9B).

**Discussion**

We used a mouse model of *Salmonella* colitis in BALB/c.D2 *Nramp1* mice, which have a wild type *Nramp1* (*Slc11a1*). Because they expressed *Nramp1* in their macrophages, they were better able to inhibit the growth of virulent *Salmonella* in their tissues, which allowed the mice to survive an infection with *S. Typhimurium* 14028s for at least 10 days. However, colitis progressed during that time and the mice developed more signs of diarrhea. Diarrhea in humans is often defined as an increase in the number and/or the volume of bowel movements per day, or the passage of unformed stools that assume the shape of the container. When it has been measured, the water content of diarrheal stools is greater than do solid stools [23],[24].

Since we could not measure the fecal output of mice directly and we could only infer the presence of unformed stools by fecal soiling of their peri-anal fur, we developed a scoring system to rate the severity of diarrhea based on the gross appearance of the colon at necropsy. A normal appearing colon containing formed feces was given a score of 1. If the cecum was shrunken and white it was scored as 2. We then turned our attention to investigating host factors that might contribute to diarrhea. *Salmonella* gastroenteritis is an inflammatory condition with large numbers of PMN in the tissues for at least the first 5 days after infection [15],[14]. Because neutrophils can make a vigorous oxidative burst and people with CGD are more susceptible to *S. enterica* bacteremia [20], we tested the role of reactive oxygen in both host defense and in mediating diarrhea. We infected mice with chronic granulomatous disease (CGD) due to a targeted mutation in *ggd* on the X chromosome [21]. We crossed homozygous *ggd* mutant females with transgenic TF6 males so that male offspring had CGD and female offspring were carriers. Both sexes inherited one allele of the *Nramp1* transgene, which is dominant, so they were IyR. After 48 hours both male and female mice had inflamed ceca (not shown) and there was no significant difference between their diarrhea scores or in the numbers of *Salmonella* that we recovered from their ceca (Figure 8). We terminated the experiment on day 7 because 1/2 of the infected males with CGD had died (none of the female carriers died). However, all the female carriers of the *ggd* mutation had full blown diarrhea by day 7 (mean diarrhea score 4.5) as happens in IyR mice, and the 3 remaining males with CGD all had diarrhea scores of 5 (Figure 8A). As expected, we recovered significantly more *Salmonella* from the male CGD mice than from the female carriers on day 7 (Figure 8B).

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Because the diarrhea score was somewhat subjective, we sought to validate it by measuring the water content of feces in the distal 5 cm of the colon, using a modification of the method of Guttman et al, who measured fecal water content from the distal colon as part of a study of the pathogenesis of diarrhea induced by *Citrobacter rodentium* [23]. For this measurement it was necessary for us to analyze the fecal material from the distal colon rather than defecated stool, since mice with diarrhea scores of 4–5 did not pass

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**Figure 2. WT *Salmonella* causes extensive colitis in BALB/c.D2 mice.** A. H&E stained section from a representative mouse showing two adjacent turns of the colon, 9 days after infection, at low magnification (40X). Note the extensive inflammation throughout the colon with many mucosal ulcers (arrows), and exudation of inflammatory cells and sloughed epithelium from opposing sides of a segment of colon merging in the lumen. B. Higher power (200X) view of the colonic mucosa from the same mouse. Note the infiltration of inflammatory cells and mucosal ulceration. There was no sub-mucosal edema in this segment but there was extensive edema in other areas of the section (see B). doi:10.1371/journal.pone.0001603.g002

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not inflamed on either day (not shown). The *phoP* mutant did not infect their spleens at either time point. In other organs the *phoP* mutants were killed.

Because *phoP* indirectly regulates *pmrA/B*, which regulates genes that modify the LPS and enhance the resistance of *Salmonella* to antimicrobial peptides such as polymyxins, we also tested *pmrA* and *pmrF* mutants in the colitis model. In contrast to the *phoP* mutant, the two *pmr* mutants were fully virulent, producing severe diarrhea (Figure 7A), multiplying in the colon, and spreading systemically (Figure 7B). The numbers of *pmrA* and *pmrF* mutants recovered from the cecum and stool were slightly greater than WT.
formed stools. Because the colon absorbs water from the feces [26], we cannot be certain that the water content of defecated feces and the water content of the fecal material in the distal colon would have been the same, had we been able to collect defecated feces from the infected mice. However, using uninfected mice the water content of defecated feces with that of feces taken from the distal 2 cm, or from the distal 3 cm of the colon (unpublished results, JF), and there was no significant difference. Thus, in normal mice the bulk of water is absorbed more proximally in the colon and that encourages us that our measurement of fecal water is a valid surrogate for the signs that are used clinically to define diarrhea.
Salmonella colitis in mice we infected them with SPI-1 (mutants. SPI-1 is required for invasion of cultured epithelial cells and the inflammatory response. Diarrhea, although we realized that diarrhea may be secondary to diarrhea before they die. Our focus was on the pathogenesis of do not survive beyond 4 or 5 days, and they do not have obvious [29], possibly because ItyS mice (such as BALB/c and C57BL/6) are a common cause of infectious diarrhea, but the S. enterica prior studies of the mouse model of mechanism of diarrhea in this infection is not well understood. We used this mouse model to begin to investigate pathogen and host factors that contribute to diarrhea due to S. enterica infections. S. enterica are a common cause of infectious diarrhea, but the mechanism of diarrhea in this infection is not well understood. Prior studies of the mouse model of Salmonella colitis have focused primarily on the pathogenesis of inflammation [16],[27],[28],[29], possibly because ItyS mice (such as BALB/c and C57BL/6) do not survive beyond 4 or 5 days, and they do not have obvious diarrhea before they die. Our focus was on the pathogenesis of diarrhea, although we realized that diarrhea may be secondary to the inflammatory response.

To begin to assess the role of bacterial virulence factors in Salmonella colitis in mice we infected them with SPI-1 (invA) or phoP mutants. SPI-1 is required for invasion of cultured epithelial cells [30] and SPI-1 mutants have been studied previously in aminoglycoside pre-treated ItyS mice. Rollenhagen and Butmann showed that SPI-1 genes are transcribed in the colon of mice with colitis [31]. Surprisingly, despite their inability to invade cultured epithelial cells, SPI-1 mutants invade the cecum as well as isogenic WT, as measured by bacteria recovered from the cecum [14]. SPI-1 mutants may be taken into the colon by mucosal dendritic cells, bypassing the epithelium [32]. Once in the submucosa where they can interact with macrophages and mesenchymal cells, inducing them to make the same panoply of pro-inflammatory molecules in a MyD88/IFN-κB dependent manner [33], they cause inflammation [15],[14],[34]. There is direct visual evidence that SPI-1 mutants localize in the lamina propria but not in epithelial cells [27]. Hapfelmeier et al used GFP expressing Salmonella with mutations in either SPI-1 or SPI-2 to localize WT and mutant bacteria in the cecum, and they found that WT Salmonella were in both epithelial cells and the lamina propria, while SPI-2 mutants were found only in the epithelial layer. Two laboratories recently reported that the SPI-2 pathogenicity island also contributes significantly to cecal inflammation [27], [16]. Hapfelmeier et al reported that the residual inflammation caused by infection with a SPI-1 mutant was reduced significantly if mice had a mutation in MyD88, which abrogates most signaling through TLRs [27]. In contrast, inflammation produced by a SPI-2 mutant (expressing an intact SPI-1) was not much reduced in MyD88 mutant mice. This implies that Salmonella can induce colonic inflammation in two ways. SPI-1 promotes epithelial invasion, which stimulates those cells to make chemokines and pro-inflammatory cytokines [35], independent of MyD88 signaling. SPI-1 mutants do not stimulate epithelial cells but they do survive inside macrophages (SPI-2 is intact) and those cells are stimulated to make cytokines and chemokines in a MyD88-dependent fashion. Which TLR is responsible for the inflammation caused by SPI-1 mutants is not known but both TLR4 and TLR5 are expressed by infiltrating macrophages [36].

We too found that WT and a SPI-1 mutant (invA) invaded the cecum and caused significant inflammation, but we found that WT Salmonella infection spread distally to infect and inflame most of the colon by day 8/9 after infection, while the SPI-1 (invA) mutant remained largely confined to the cecum, as did the pathology. Most importantly, the diarrhea scores and the water content of the feces from mice infected with the invA mutant were significantly lower than the scores produced by WT Salmonella (Figure 3). The localization of inflammation produced by invA to the cecum may explain why invA did not cause significant diarrhea in this model of colitis. It is not clear why the cecum was more susceptible to invA infections than the rest of the colon. The terminal ileum enters the cecum and that part of the colon is patulous, which may allow more time for the bacteria to be in contact with the mucosa. Alternatively, there may be differences in the residual flora of the cecum and the rest of the colon, though we did not study that. invA Salmonella may also be taken up by M cells that overlie Peyer’s patches, which are present in the cecum. Hoever, Hapfelmeier et al [27] showed that WT and SPI-1 mutants can invade the ceca of LTβR -/- mice, which have no Peyer’s patches or GALT, so we cannot attribute susceptibility to the lymphoid aggregates that normally are in the mouse cecum.

We also studied a phoP mutant in this model. PhoP mutants do not survive inside macrophages [37]. PhoP/Q is a two component transcriptional regulator that regulates expression of many Salmonella genes, including genes that modify LPS and make the bacteria more resistant to antimicrobial peptides [38]. A phoP mutant was nearly avirulent in our model (Figure 4). The mutant infected the cecum and caused localized inflammation, increased
fecal water content, and mild diarrhea by day 2 after infection (Figure 4), as might be expected since the phoP mutation may even enhance epithelial cell invasion in vitro [39]. However, BALB/c.D2 mice rapidly reduced the numbers of Salmonella over the next week. The rapid clearance of phoP- bacteria from the colon was reflected in markedly reduced inflammation in the colon; on day 2 after infection, the diarrhea score was significantly higher than that of uninfected control mice and not significantly different from the water content of mice infected with WT Salmonella (14028s). However, by day 8 the water content of feces from mice infected with WT had increased to nearly 80% while the water content of feces from phoP- infected mice had returned to normal. C. Comparison of the numbers of CFU of WT and phoP Salmonella on days 2 and 7 after infection. Geometric means±SEM are shown. * = P < 0.05, ** = <0.001. P values for the differences between means were calculated using the Mann-Whitney test. There were 6 mice/group. We did not calculate P values for spleen since there were <10 CFU of the phoP mutant, or for the CFU in stool on day 8 since we had only 3 stools from WT mice for culture.

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Figure 5. A phoP mutant of 14028s invades the colon, but does not persist in the colon, and does not cause colitis in BALB/c.D2^{Nramp1} congenic mice. A. Diarrhea scores for phoP-infected mice on days 2 and 8/9 after infection. The score is significantly higher on day 2 (P<0.05). B. Water content of the stool on those days. The water content of the stool of phoP-infected mice on day 2 after infection was significantly higher than that of uninfected control mice and not significantly different from the water content of mice infected with WT Salmonella (14028s).
after infection there was no evidence of diarrhea and no inflammation in the cecum (Figure 4E). Thus, phoP mutant Salmonella could invade but could not survive in the colon, and they did not cause ongoing inflammation or a high diarrhea score, and they caused only a transient increase in fecal water content. We conclude from this that epithelial cell invasion by Salmonella is not sufficient to produce diarrhea in this model, though it may transiently impair epithelial cell function enough to decrease net water absorption.

PhoP/Q regulates many genes and it is not clear which of them are necessary for virulence. Among the genes that are indirectly regulated by PhoP/Q are pmrA/B [40], another two component regulatory system that controls a subset of the genes (pmrF operon) that S. enterica use to modify the negative charge on their LPS to increase resistance to antimicrobial peptides such as polymyxin B [41]. Therefore, we tested pmrA and pmrF mutants for their ability to cause colitis, and they were fully virulent. Thus, the profound attenuation of a phoP/Q mutant in the colitis model is not due to lack of expression of any of the pmrA-coregulated genes. PmrA/B control only a subset of the genes that regulate modifications of the LPS, and pmrA mutants remain resistant to some antimicrobial peptides such as cathelicidin. phoP mutants are more susceptible to cathelicidins, which may explain why phoP mutants are avirulent in this model [42].

The above results suggest that inflammation was connected with diarrhea. If inflammation is somehow responsible for diarrhea in...
Female B6.gp91phox -/- mice with male TF6 (Nramp1 G169mice on an ItyR genetic background by crossing homozygous

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stool, and only 2/6 females had solid feces to culture.

no solid feces in the colons of CGD mice so we could not culture their

two but significantly higher in the CGD mice on day 7 after infection.

significantly different on day 7, although there were only 3 surviving CGD

controls on day 2 (P<0.05), but the scores for the two strains were not

significantly different on day 7, although there were only 3 surviving CGD

mice on day 7. B. Geometric mean CFU±SEM for various tissues on days 2

and 7 after infection. Colony counts were similar in the two groups on day

two but significantly higher in the CGD mice on day 7 after infection.

* indicates a P value (Mann Whitney U test) of <0.01. On day 7 there were

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Salmonella gastroenteritis, we reasoned that inflammatory media-
tors made by the host may contribute to diarrhea. We assessed the

contribution of one host factor, reactive oxygen species (ROS), by

infecting gp91phox deficient mice, which cannot generate ROS. Since the gp91phox mutation is on a B6 genetic background, and

gp91phox is an X-linked gene, we were able to breed gp91phox -/- mice on an ItyR genetic background by crossing homozygous female B6 gp91phox -/- mice with male TF6 (Nramp1G169

transgenics). We then compared male F1 mice that had CGD with female littermates, who were carriers of the mutation and did not have CGD because of the Lyon effect. We found that male mice with CGD developed severe diarrhea when infected with 14028s, as severe as, or possibly worse than the controls. These results show that ROS from the host are not necessary to produce diarrhea. Not surprisingly, the male mice with CGD were also much more susceptible to Salmonella infection than were their sisters. These results extend the observations of Shiloh et al who reported that B6 mice with CGD are more susceptible to systemic Salmonella infections [43]. We also used the ItyR gp91phox -/- mice to determine whether CGD mice were susceptible to oral infection with the phoP mutant, and they were not. This result implies that ROS are not required to kill phoP mutant Salmonella in vivo, and that there are non-oxidative killing mechanisms in mice that are an effective defense against phoP mutants but not WT Salmonella.

While we were doing these experiments Stecher et al reported that 2 strains of genetically resistant mice (ItyR) developed a severe form of Salmonella colitis, though the analysis presented was only of the cecal pathology [28]. In their experiments DBA/2 mice died of a progressive systemic infection, but the BALB/c.D2Nramp1 congenic mice that we studied did not die of infection. We are still uncertain why BALB/c.D2Nramp1 congenic mice died, but they probably died from colitis and not from overwhelming systemic Salmonella infection. In addition to Nramp1, there are a number of polymorphic genes in mice that influence resistance to systemic Salmonella infections [44], and these could explain the difference between the course of infection in BALB/c.D2Nramp1 congenic mice and the strains studied by Stecher et al.

In summary, we have developed a model of Salmonella colitis that mimics a severe form of human Salmonella gastroenteritis that is manifested as dysentery [45],[46],[47]. The infected mice showed several features of diarrhea and can be used to study the pathogenesis of diarrhea in mice. We found that neither SplI mutant Salmonella that are unable to invade epithelial cells, nor phoP mutants that cannot survive in macrophages are able to cause diarrhea, and they caused much less inflammation in the colon than do wild type Salmonella. Diarrhea occurred even in the absence of the host’s oxidative burst.

Methods

Mice

C57BL/6 (B6) and B6.gp91phox -/- mice were purchased from Jackson Laboratory (Bar Harbor, MA). B6. Nramp1G169 transgenic mice (TF6) [48] and BALB/c.D2Nramp1 congenic mice [49] were bred at our institution under SPF conditions. In most experiments we included mice of both sexes and matched them for sex in all groups. We crossed female B6.gp91phox -/- with male B6. Nramp1 transgenic mice in order to breed F1 males that carried the mutant B6.gp91phox allele, and therefore have chronic granulomatous disease (CGD) on a Nramp1G169 genetic background, since the WT version of Nramp1 is dominant. The female F1 mice are carriers of the B6.gp91phox mutation and served as the controls for the male mice.

Infection

We administered oral kanamycin (40mg) in 0.1 cc of saline by gavage. The following morning the mice fasted for 4 hours before we infected them by gavage with ~5x10^5 CFU of S. Typhimurium 14028s in 0.1 ml of 0.1M NaHCO_3. Mice were allowed free access to food and water. They were weighed daily in some experiments. At necropsy we exposed the abdominal cavity and scored the severity of the diarrhea before we disturbed the tissues. We processed the tissues for culture as previously described [50], except for the cecum and feces. If there were formed feces in the distal colon, or if the mouse passed a formed stool when being removed from the cage, that was weighed and then homogenized in sterile saline and then diluted in saline for quantitative culturing on Hektoen Enteric (HE) agar with 20 μg/mL of kanamycin added. Each cecum was bisected and half used for culture after rinsing out the contents and then soaking the tissue in gentamicin 20mg/L for 30 minutes at room temperature. We then rinsed
Figure 9. Mice with CGD are not more susceptible to oral infection with a *phoP* mutant *Salmonella*. A. Mean±SD of diarrhea scores of mice with CGD and controls (female littermates), 2 and 8 days after infection. There was no significant difference between the diarrhea scores of the two groups on either day. B. Quantitative bacteriology results for mice with CGD and control. There were no significant differences between CFU recovered from mice with CGD and littermate controls on either day. Note that by day 8 there were only ~200 CFU recovered from the ceca of both groups, and there were <10 CFU recovered from spleen cultures, indicating that in vivo the NADPH oxidase was not involved in the highly effective innate immune response that can resolve an oral infection with *phoP* mutants.

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**Table 1.** Scoring system for grading diarrhea.

| Grade | Criteria |
|-------|----------|
| 1     | normal colon with normal formed feces in the distal colon or rectum |
| 2     | cecum that is smaller than normal with areas of white and/or gas bubbles in the lumen; the rest of the colon is normal |
| 3     | severe typhlitis (white, shrunken cecum) with involvement of the proximal colon so that it is fluid filled; formed stools in distal colon |
| 4     | Grade 3 plus fluid throughout most of the colon with soft feces in the distal colon |
| 5     | no formed feces in the entire colon and the cecum white and shrunken; colon filled with clear or bloody mucus. |

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away the gentamicin with iced saline prior to grinding the tissue. All the tissues were homogenized mechanically in 1 ml of saline. The tissue slurry was serially diluted in sterile saline, and 100 μl of appropriate dilutions were cultured in duplicate on trypticase soy agar plates (spleens and mesenteric lymph nodes) or HE agar with 20 μg/ml of kanamycin added. All lactose-negative H₂S positive colonies that grew on the HE-kanamycin plates were assumed to be Salmonella. Three cm long segments of the colon were processed similarly before they were cultured. If mice died before the time of sacrifice we assigned them the same number of colonies.

The scoring system that we used to assess the severity of the diarrhea is shown in Table 1. To determine the water content of the feces we removed all the luminal contents from the distal 3 cm of the colon and placed them into Eppendorf tubes. The tubes with the lids off were weighed and then dried in a SpeedVac (Thermo Savant, Waltham, MA) for 4–6 hours until all samples were dry. The tubes were reweighed and the percentage decrease in weight was calculated. This method is a modification of the one used by Gutman et al. to measure water content of feces of C. rodentium-infected mice [25].

Bacteria

We constructed derivatives of Typhimurium strain 14028s containing a kanamycin resistance cassette (kanR) inserted in a genetically silent region downstream from the pmrA operon in the virulence plasmid. This S. enterica serovar Typhimurium 14028s KanR strain [51] was used to allow the bacteria to survive in the colon of mice treated with oral kanamycin. S. Typhimurium 14028s in1428::aphT has been described previously [52]. The phoP::Tn10 allele [19] was transduced into the 14028s KanR strain using phage P22, as described [51]. pmrA [41] and pmrF [53] mutants were transduced into the 14028s KanR strain as above. Bacteria were grown overnight in TSB, and washed and resuspended as previously described [54].

Histology

We filled the entire colon with Bouin’s solution, tightly coiled the colon around a wooden applicator stick, and immersed the coiled colon in Bouin’s solution. After embedding in paraffin we made 5 μm thick sections that we stained with hematoxylin and eosin (H&E). Each slide was coded and read blindly by the PI.

Statistics

Results were analyzed using GraphPad Prism version 4 (San Diego, CA). Multiple groups were compared using a one way ANOVA and the differences between two groups were examined with the Kruskal-Wallis statistic. To compare two groups of results we used the non-parametric Mann-Whitney U test. P values of <0.05 were considered significant.

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Author Contributions

Conceived and designed the experiments: JF. Performed the experiments: SO HW. Analyzed the data: JF. Contributed reagents/materials/analysis tools: DG JG. Wrote the paper: DG JF.

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