A Mouse Model of Persistent Salmonella Infection (13-Nov-2004)

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Introduction

When a pathogenic microorganism first infects its host there is usually a dramatic engagement with the innate and adaptive immune systems that may result in disease symptoms. If the microbe and the host survive this initial interaction, the adaptive host immune system usually clears the invading offender. However, some pathogenic bacteria are capable of maintaining infections in mammalian hosts even in the presence of inflammation, specific antimicrobial mechanisms, and a robust adaptive immune response, which will serve as our definition of a persistent infection. For example, Salmonella typhi causes systemic infection (typhoid fever) that involves colonization of the reticuloendothelial system (RES). Some S. typhi infected individuals become life-long carriers, periodically shedding high numbers of bacteria in their stools. Persistently infected carriers serve as the reservoir of these pathogens and the carrier state is an essential feature for survival within a restricted host population.

Most often, persistent colonization with Salmonella is clinically unapparent. However, even in the absence of clinical symptoms, infection poses some risk to the host. Humans carrying S. typhi are at an increased risk of developing hepatobiliary cancer. The long-term residence of the bacteria in a privileged host niche poses several fundamental biological questions. What is the replicative and metabolic state of the bacteria during persistent asymptomatic infection? How do these organisms manage to escape clearance for so long in the presence of a host response? We are only now beginning to understand the bacterial and host factors in this persistent host-microbe interaction and the answers will likely provide new and exciting directions for research inquiry in the fields of microbial pathogenesis and immunology.

We will describe here a mouse model of persistent Salmonella infection that we have characterized in our laboratory [1]. The host immune factors involved in Salmonella persistence are beginning to be understood. We have also been identify Salmonella genes involved in persistence and will describe in detail one of these genes, mig-14 [2].

Model of Chronic S. typhimurium Infection in 129sv Mice

We developed a long term infection model in mice to study the pathogenesis of a persistent Salmonella infection in a mammalian host. A previous study found that a strain of S. enteritidis was carried in the spleens of 129sv mice for 42 weeks following an intravenous inoculation with 10^7 organisms. We inoculated 35 129sv mice orally with 1x10^8 CFUs of a wild-type S. typhimurium strain, SL1344, and monitored tissue colonization fecal shedding and tissue pathology over the course of a year.

Most of the mice survived this dose of S. typhimurium. At early times post infection most of the mice had histopathological lesions characteristic of S. typhimurium in the spleen, liver, Peyer’s patches (PP) and mesenteric lymph nodes (MLN), indicative of an acute infection. The number and severity of lesions and the weights of infected spleens decreased with time, indicative of convalescence. Three mice died over the course of the experiment, on 165, 280, and 295 days post infection, and at postmortem, these mice cultured positive for S. typhimurium.

S. typhimurium infection of 129sv mice induced pronounced splenomegaly and enlarged mesenteric lymph nodes, which peaked after 30 to 40 days and persisted for several weeks. Spleen sizes slowly returned to a more normal size, however there was a small, but significant increase in spleen weight compared to age-matched uninfected control spleens. Most of the mice did not display any signs of illness such as malaise, weight loss and ruffled fur despite the chronic presence of bacteria. At 60
days, we found that the mice were still colonized at systemic sites. Although all 5 of the mice sacrificed at 60 days were colonized, the bacterial burden per gram of tissue was considerably lower than mice infected for 5 days. We found some variation in the presence of *S. typhimurium* in the various tissues. All of the mice were colonized in the MLN at 60 days and 2 mice were colonized in all of the tissues tested. When we monitored the feces for the presence of *S. typhimurium*, we found that 3 mice were shedding *S. typhimurium* in the feces on day 60; the presence of *S. typhimurium* in the feces correlated with gall bladder and cecal colonization. When we monitored additional mice for fecal shedding on 4 consecutive days (114, 115, 116, 117 days post infection) we found that shedding varied from day to day in individual mice, suggesting that shedding occurs in waves as *Salmonella* replicates.

At 140 and 180 days post infection, we did not notice an increase in the overall bacterial burden per gram of tissue (2 to 3 logs per gram tissue), however the numbers of mice that were colonized at intestinal sites, cecum and PP, decreased. In contrast, all of the mice were colonized in the reticuloendothelial system, MLN and/or spleen. One mouse was shedding at 180 days and was colonized in the cecum and gall bladder.

By 270 days, 4 of the 5 mice were persistently infected in the MLN and one mouse appeared to have cleared the infection. The overall bacterial burden in the MLN did not change dramatically compared to the earlier time points. By 365 days, 5 of the remaining 13 mice had cleared the infection and one mouse had apparent signs of illness and was colonized by *S. typhimurium* in all tissues tested. All 8 of the persistently infected mice contained *S. typhimurium* in the MLN, with a range of 2 to 5 logs CFU per gram tissue. Taken together these data indicate that after the early or acute phase of a systemic *Salmonella* infection that *S. typhimurium* persists in the MLN and/or spleen of chronically infected mice.

**Histology of Chronically Infected Tissues**

Histopathological lesions were more frequent in the spleen, liver and mesenteric lymph nodes at 60 days post oral inoculation than at 365 days. The tissues from mice infected for 60 days contained typical foci of necrosis, microgranulomas, or accumulations of PMNs. At 140 days, discreet lesions were scarce in the liver and spleen. These rare inflammatory foci consisted predominantly of macrophages with minimal central necrosis. By 365 days, the focal granulomas were not significantly different than 140 days, but they tended to have increased lymphocytes and large mononuclear cells surrounding central areas of tightly opposed macrophages.

**S. typhimurium Persist in MOMA-2· MLN Macrophages**

The tissue colonization data of 129sv mice orally infected with *S. typhimurium* indicated that during the initial stages of infection high numbers of bacteria are found at sites throughout the mouse, but were beginning to be cleared between 60 and 270 days post infection. The bacteria then persisted in a limited number of sites, predominately the MLN, despite a strong antibody response.

We wished to determine the site of *S. typhimurium* persistence in the MLN. To ensure that MLN were taken from mice that were still infected with wild-type *S. typhimurium*, we monitored the course of infection in 129sv mice using a noninvasive method that detects bacterial signal in live animals, an In Vivo Imaging System (IVIS). Mice were infected with a wild-type strain of *S. typhimurium*, SMB500, that constitutively expresses the genes necessary for bioluminescent light production. This strain is as virulent as wild-type SL1344 and retains the bioluminescent genes in the mouse infection model. By following the course of infection in this noninvasive manner, we found that some mice would vary in the level of bacteria over time. For example, figure 6 shows a mouse that has a higher bioluminescent signal at 23 days than at 40 days. At 80 days post-infection, the signal increased again. Three mice that were infected with *S. typhimurium* for 80 days and had elevated bioluminescent signals were sacrificed and the MLN from each mouse were frozen and sectioned for immunohistochemistry.

To determine the host cell(s) that *S. typhimurium* are associated with in persistently infected MLN, we stained frozen sections with several antibodies to host immune cells. Initially we stained sections with an antibody that recognizes neutrophils, Gr-1 (Pharmingen), an anti-Salmonella antibody and an antibody that non-specifically stains the host cells. We found that bacteria did not colocalize with neutrophils, rather they resided within large host cells that were surrounded by neutrophils. This finding and the results of our histological stainings led us to examine the remaining sections with an antibody that stains the cytoplasm of all macrophages, MOMA-2 (Serotec). The bacteria were mainly found colocalized with MOMA-2· macrophages in macrophage-rich areas of the lymph nodes within the subcapsular sinus and outer cortex.

Further analysis of the z-stacks obtained on the confocal microscope revealed that bacteria were inside these macrophages, as seen in the xz, yz and 3D projections. To determine the frequency with which *S. typhimurium* were located inside of macrophages, we counted every bacterium found within all sections and scored whether they were inside or outside a
MOMA2+ host cell. 82% of the bacteria were clearly intracellular, while the remaining bacteria were either extracellular or their location was unclear. We noted at the same time the number of bacteria residing within the infected host cell and found that the average number was between 3 and 4 bacteria. We further characterized the host immune cells in the foci of bacterially-infected macrophages and found that in addition to macrophages, there were predominantly B lymphocytes in the immediate vicinity of the infected macrophages. Thus, S. typhimurium persist intracellularly within macrophages of the MLN of 129sv mice.

**IFN-γ Signaling Limits Replication of mig-14 Mutant Bacteria in Mouse Infection**

Previous work demonstrated that mutation of mig-14 leads to an inability of S. typhimurium to replicate within the spleen and liver of infected mice at later times post-infection. Significantly higher amounts of serum IFN-γ were also present in mig-14 mutant infected mice compared to wild-type infected mice on day 7 post-infection. It has been reported that peak production of IFN-γ by CD4+ and CD8+ T cells occurs at day 7-14 post-infection with vaccine strains of S. typhi. We therefore hypothesized that the inability of mig-14 mutant bacteria to replicate in RES organs at this time might be due to the proinflammatory effects of IFN-γ and other cytokines secreted as a result of adaptive immune responses. We compared the infection of C57Bl/6j and isogenic IFN-γ/-/ mice with wild-type and isogenic mig-14 mutant bacteria to examine the role of IFN-γ in controlling the replication of the mig-14 mutant.

As was previously reported with infection of BALB/c mice, we saw virtually no difference in the CFU of wild-type and mig-14 mutant bacteria obtained from the spleen and liver of C57Bl/6 mice at day 5 post-infection. Also consistent with our previous observations, we saw significantly fewer mig-14 mutant bacteria in these tissues at days 7 and 9 post-infection than SL1344 bacteria, indicating that mig-14 is required for replication in systemic tissues of wild-type mice at later times post-infection.

In contrast, in IFN-γ-/- mice at day 5 post-infection, the levels of SL1344 were 10-fold higher than the amount of bacteria present at day 9 post-infection of C57Bl/6 mice, consistent with observations demonstrating a clear role of IFN-γ in controlling replication of intracellular bacterial pathogens during infection. At day 7 post-infection, both mig-14 mutant-infected and SL1344-infected mice had extremely high levels of bacteria present in the tissues, and the infectious burdens of both wild-type and mig-14 mutant bacteria were identical. These data indicate that IFN-γ signaling is crucial for controlling the replication of mig-14 mutant bacteria. However, we found that at day 5 post-infection, the levels of mig-14 mutant bacteria in the tissues of mice that had not died were significantly lower than those of SL1344, indicating the possibility that an IFN-γ independent mechanism controls the replication of mig-14 bacteria in these mice initially, but cannot contain the infection by these mutant bacteria in the absence of IFN-γ.

A significant number of IFN-γ-/- mice infected with either SL1344 or mig-14 mutant bacteria died due to the overwhelming replication of bacteria before the organs could be analyzed for CFU counts. The survival curves of the SL1344 or mig-14 infected mice were virtually superimposable, indicating that the absence of IFN-γ, mig-14 mutant bacteria rapidly replicate in systemic organs and kill infected mice indistinguishably from wild-type bacteria.

**mig-14 Contributes to Salmonella Persistence in Host Tissues**

We challenged 129Xi/SvJ by oral infection with 1×107 colony forming units of SL1344 or mig-14 mutant bacteria. At various times post-infection, mice were sacrificed and the bacterial load present in each tissue examined. At day 28 post-infection, there was no statistically significant difference between mice infected with any of the bacterial strains, as was the case for all of the timepoints prior to day 28. Since persistently infected mice carry the highest bacterial burdens in the mesenteric lymph nodes, we focused our analysis of these mice on the spleen and MLN. In the MLN at day 123 post-infection, 5 mig-14 infected mice had cleared the bacteria, whereas only 2 SL1344 infected mice had cleared the infection. Of the mice that had not cleared the infection, the MLN of mig-14 infected mice contained significantly lower amounts of bacteria compared to SL1344 infected mice, although there was not a substantial difference in the number of mice that had bacteria present in the MLN. In the spleen, we observed that although the bacterial counts were uniformly lower than in the MLN, there was not a significant difference in colony counts between mutant and wild-type infected mice. However, significantly fewer mice still harbored mig-14 bacteria in the spleen compared to the spleens of SL1344 infected mice. These data suggested that mig-14 is important in long-term maintenance of the persistent infection. Since the majority of persisting S. typhimurium reside within macrophages in the RES, we examined whether the decrease in the ability of mig-14 mutant bacteria to persist in mice correlated with a decrease in bacterial survival within macrophages.

**mig-14 Promotes Salmonella Replication in Activated Macrophages**

The observation that control of mig-14 mutant bacterial replication in the spleen and liver subsequent to day 5 post-infection was dependent on IFN-γ indicated that host inflammatory responses are necessary to control replication of mig-14 mutant bacteria. Macrophages activated by IFN-γ upregulate a variety of effector functions including production of reactive oxygen
and nitrogen species, increased expression of antimicrobial peptides, and increased trafficking of phagocytosed material to the lysosome. We therefore examined the ability of mig-14 mutant bacteria to survive and replicate in either unactivated or activated cells of the RAW 264.7 macrophage-like cell line.

*mig-14* mutant bacteria replicated in unactivated RAW274.7 macrophages indistinguishably from SL1344 wild-type bacteria. Both SL1344 and mig-14 mutant bacteria showed an approximately 10 - 15 fold increase in colony forming units (cfu) over the infection time-course. *virK* is located immediately upstream of *mig-14* on the *Salmonella enterica* genome, and shares a number of characteristics with *mig-14*, including PhoP-dependent regulation and a role in resistance to antimicrobial peptide killing. We therefore examined the ability of *virK* mutant bacteria to survive and replicate within host macrophages and found that, like *mig-14*, *virK* mutant bacteria replicated to the same level as wild-type bacteria in unactivated macrophages. In contrast to both *mig-14* and *virK* mutants, mutation of *phoP* caused complete loss of *Salmonella*’s ability to replicate within unactivated macrophages, consistent with previous studies demonstrating the requirement of *phoP* for intracellular replication.

**mig-14 and virK are Required for Resistance to the Murine Antimicrobial Peptide CRAMP**

Recently published studies demonstrated that a mouse homologue of the human cathelicidin LL-37, designated CRAMP [3], is expressed in murine macrophages and colocalizes with the *Salmonella* containing vacuole [4]. These studies also observed a role for *phoP* in resistance to the effects of CRAMP. We had previously observed that *mig-14* and *virK* mutant bacteria are more susceptible to killing by several different antimicrobial peptides in vitro. We therefore examined whether *mig-14* and *virK*, both of which are *phoP* regulated genes, might be necessary for *S. typhimurium* resistance to CRAMP. We found that both *mig-14* and *virK* mutants were significantly more sensitive to killing by CRAMP peptide than the wild-type SL1344 strain when the bacteria were grown in nutrient-limiting acidic media prior to the antimicrobial peptide killing assay.

**mig-14 and virK Inhibit Binding of CRAMP Peptide to the Bacterial Surface**

To further investigate the role of *mig-14* and *virK* in antimicrobial peptide resistance by *S. typhimurium*, we conjugated synthetic CRAMP to fluorescein isothiocyanate (FITC) and analyzed the association of FITC-CRAMP with different *S. typhimurium* strains by flow cytometry. SL1344, *mig-14*, *virK* and *phoP* mutant bacteria expressing a rapidly maturing DsRed protein were incubated with FITC-CRAMP for 30 minutes in a low osmolarity buffer prior to FACS analysis. Intact bacteria were identified based on side-scatter and level of DsRed fluorescence.

In comparison to wild-type bacteria, all the mutants demonstrated a significant shift toward higher staining. The *mig-14* and *virK* mutants also demonstrated a bi-modal distribution, with a significantly greater proportion in the high-staining population (61% and 67%, respectively) compared to 10.5% of SL1344. In contrast to SL1344, neither the *mig-14* nor *virK* mutants had any bacteria in the low-staining population. The *phoP* mutant showed only a single peak with virtually the entire population exhibiting a high level of FITC staining. These percentages correlate well with the degree of sensitivity of these mutant bacteria to killing by CRAMP peptide.

**Summary**

*Salmonella enterica* serovar Typhimurium infects a wide variety of mammalian hosts and in rodents causes a typhoid-like systemic disease involving replication of bacteria inside macrophages within reticulo-endothelial tissues. Previous studies demonstrated that the *mig-14* and *virK* genes of *Salmonella enterica* are important in bacterial resistance to antimicrobial peptides and are necessary for continued replication of *S. typhimurium* in the liver and spleen of susceptible mice following orogastric inoculation. In this work we report that inflammatory signaling via IFN-γ is crucial to controlling replication of *mig-14* mutant bacteria within the liver and spleen of mice after oral infection. Using a *Salmonella* persistence model recently developed in our laboratory, we further demonstrate that *mig-14* is necessary for long-term persistence of *Salmonella* in the spleen and mesenteric lymph nodes of chronically infected mice. Both *mig-14* and *virK* contribute to the survival of *Salmonella* in macrophages treated with IFN-γ and are necessary for resistance to CRAMP, an antimicrobial peptide expressed at high levels in activated mouse macrophages. These data indicate that antimicrobial peptide resistance is necessary not only during the initial stages of breaching host barriers, but also contributes to survival of *Salmonella* within macrophages in systemic sites during both acute and persistent infection.

**References**


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