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Early eradication of persistent *Salmonella* infection primes antibody-mediated protective immunity to recurrent infection¹

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Abstract

Typhoid fever is a systemic, persistent infection caused by host-specific strains of *Salmonella*. Although the use of antibiotics has reduced the complications associated with primary infection, recurrent infection remains an important cause of ongoing human morbidity and mortality. Herein, we investigated the impacts of antibiotic eradication of primary infection on protection against secondary recurrent infection. Using a murine model of persistent *Salmonella* infection, we demonstrate protection against recurrent infection is sustained despite early eradication of primary infection. In this model, protection is not mediated by CD4⁺ or CD8⁺ T cells because depletion of these cells either alone or in combination prior to rechallenge does not abrogate protection. Instead, infection followed by antibiotic-mediated clearance primes robust levels of *Salmonella*-specific antibody that can adoptively transfer protection to naïve mice. Thus, eradication of persistent *Salmonella* infection primes antibody-mediated protective immunity to recurrent infection.

Keywords

SALMONELLA; ANTIBIOTIC TREATMENT; RECURRENT INFECTION

1. Introduction

Typhoid fever is a persistent, systemic infection caused by host-adapted strains of Gram-negative bacteria within the *Salmonella* genus. The development and use of antimicrobials with bactericidal activity against *Salmonella* has transformed this once debilitating and often fatal infection into a readily treatable condition. Unfortunately even with antibiotic therapy, recurrent disease occurs in 5 to 15% of individuals [1–4]. Molecular genotyping and phenotyping of *S. enterica* serotype Typhi (*S. typhi*) isolates from individuals with primary and recurrent infection suggest recurrence may be caused by re-activation of latent or secondary infection [2,5,6]. However, for individuals living in endemic areas where re-exposure is essentially unavoidable, protection from recurrent infection is paramount, while the significance of distinguishing *Salmonella* isolates associated with re-activation or secondary infection appears less relevant.

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Regardless of the specific etiology, the clinical symptoms of recurrent compared with primary infection are less severe and of shorter duration [7]. Similarly, reduced rates of clinical typhoid fever and infection relapse have been reported for human volunteers previously recovered from typhoid compared with naïve individuals after challenge with virulent *Salmonella* [8], and reduced attack rates occur for individuals with prior *Salmonella* infection during an outbreak among military personnel exposed to infected food handlers [9]. These epidemiological features of human typhoid suggest naturally-acquired *Salmonella* infection confers some protection against secondary infection.

Protection from recurrent disease triggered by primary infection is also reproduced in animal models of *Salmonella* infection. For example, natural recovery from experimental typhoid fever protects chimpanzees from fever, bacteremia, and systemic inflammation after secondary challenge with virulent *Salmonella* [10]. For mouse typhoid caused by *S. enterica* serotype Typhimurium (*S. typhimurium*) infection, primary infection with live attenuated *Salmonella* mutants confers a high level of protection against secondary challenge with virulent *Salmonella* [11,12]. Thus, animal models of typhoid infection allow the potential impact of antibiotic treatment in priming protective immunity to be more precisely characterized. In this regard, a recent study reported sharply reduced protection against recurrent infection after early eradication of primary infection with virulent *Salmonella* compared with that primed by an attenuated *Salmonella* mutant that causes more sustained infection [13]. These findings suggest antimicrobial therapy, while beneficial for curtailing the sequelae of primary infection, may also blunt the priming of protective immunity conferred by natural infection. However, the inherent susceptibility C57BL/6 mice lacking the resistant allele of *Nramp1* to virulent *S. typhimurium* used in this study required the eradication of primary infection within two days. Therefore, the effects of antibiotic-mediated clearance of primary infection during the later and persistent phase of this infection remain undefined. In this study, mice containing the resistant allele of *Nramp1* that develop persistent infection with virulent *Salmonella* were used to investigate the impacts of primary infection eradication on protection against secondary *Salmonella* infection.

2. Materials and methods

2.1. Mice

C57BL/6 and 129SvJ mice were purchased from the National Cancer Institute. B6.129 F1 mice generated by intercrossing C57BL/6 females with 129SvJ males as a model for persistent infection with virulent *Salmonella* has been described [14–16]. All mice were generated and maintained in specific pathogen-free facilities and used between 6–8 weeks of age. These experiments were conducted under University of Minnesota IACUC approved protocols.

2.2. Bacteria, infections, and antibiotic treatment

The virulent *S. enterica* serotype Typhimurium (*S. typhimurium*) strain, SL1344, has been described [12,17,18]. For infections, *S. typhimurium* was grown to log phase in brain heart infusion (BHI) media at 37 °C, washed and diluted with saline and injected intravenously through the lateral tail vein [16]. The number of recoverable *Salmonella* CFUs was quantified by plating serial dilutions of organ homogenates onto BHI agar plates. Where indicated, enrofloxacin was added to the drinking water (2 mg/ml) beginning five or twenty days post-infection. Mice were withdrawn from antibiotics for at least five days prior to secondary infection. For re-challenge, 1×10^4 or 1×10^6 CFUs of SL1344 was injected intravenously. Heat-killed *Salmonella* was prepared by resuspending SL1344 in sterile saline and incubating at 75°C for 60 minutes, and plating to confirm the absence of live bacteria as described [19].

2.3. Reagents for cell staining, antibody ELISA, and cell depletion

Antibodies and other reagents for flow cytometry and ELISA were purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA). For ELISA, flat bottom 96-well plates were coated with 1.25×10^7 CFUs heat-killed SL1344 diluted in 0.1 M NaHCO₃ and incubated overnight at 4°C. Wells were then blocked with 1% albumin, assayed with serial dilutions of serum from *Salmonella* infected mice followed by biotinylated anti-mouse isotype specific antibodies, and developed with streptavidin conjugated to peroxidase and *O*-phenylenediamine substrate. For CD4⁺ and CD8⁺ T cell depletion, 500 µg of purified anti-mouse CD4 (clone GK1.5) and/or anti-mouse CD8 (clone 2.43) antibody (BioXCell) were inoculated intraperitoneally one day prior to *Salmonella* infection. For transfer, serum was collected from donor mice, and transferred intravenously into naïve recipient mice (350 to 400 µl/mouse) one day prior to *Salmonella* infection.

2.4. Statistics

The difference in number of recoverable bacterial CFUs and survival were evaluated using the Student's *t* and log-rank tests, respectively (GraphPad, Prism Software) with $P \leq 0.05$ taken as statistically significant.

3. Results

3.1. Protective immunity despite early eradication of primary *Salmonella* infection

The reduced severity, shorter duration, and lowered attack rates of recurrent compared with primary *Salmonella* infection in humans suggest natural infection primes some protective effects against secondary infection [8,9]. To address how eradication of primary infection impacts these protective effects, we compared the susceptibility against secondary recurrent infection with virulent *Salmonella* for mice treated with antibiotics after primary infection or control mice without primary infection. We initially enumerated the relative susceptibility for *S. typhimurium*-infected mice (10^4 CFUs) treated with enrofloxacin beginning day 20 after primary infection. Enrofloxacin is a fluoroquinolone antibiotic, and this dose has been shown to eradicate systemic *Salmonella* infection within five to seven days after administration in the drinking water [13,20]. Using this approach, highly significant (≥ 50 –100-fold) reductions in recoverable *Salmonella* were found after secondary challenge (10^4 CFUs) for mice eradicated of primary infection compared with control mice without primary *Salmonella* infection (Fig. 1A). This reduction in number of recoverable *Salmonella* CFUs was not due to residual enrofloxacin because both groups of mice were treated and withdrawn from this antibiotic in parallel. Thus, protection against recurrent *Salmonella* infection is sustained despite eradication of primary infection.

To investigate if earlier eradication of primary *Salmonella* infection would impact protection against recurrent infection, we compared the effects of antibiotic initiation beginning day 5 compared with day 20 after primary infection. We found protection against secondary infection was sustained with eradication of primary infection beginning day 5, and these protective effects were indistinguishable compared with mice treated with antibiotics later (day 20) (Fig. 1B). Importantly, these protective effects could not be attributed to reduced efficiency of antibiotic eradication of virulent *S. typhimurium* that causes persistent infection in mice containing the resistant allele of *Nramp1* because enrofloxacin readily eradicated primary infection within the first 5–7 days after treatment (Fig. 2).

Additional experiments explored the magnitude of these protective effects by enumerating survival following secondary infection with a higher dose of virulent *S. typhimurium* (10^6 CFUs) that normally causes lethal infection even in mice containing the resistant allele of *Nramp1*. These experiments revealed that eradication of primary infection with antibiotics

beginning day 5 was sufficient to protect mice from lethal *Salmonella* infection, while naïve control mice treated with antibiotics in parallel all succumbed within the first week after infection (Fig. 3). These protective effects of primary infection followed by eradication with antibiotics shortly thereafter were specific to live *Salmonella* infection because the same dose of heat-killed *Salmonella* used for primary infection (10^4 CFUs) provided no protective effects (Fig 3). These results demonstrate primary *Salmonella* infection even with antibiotic eradication primes protection against recurrent infection.

3.2. Neither CD4⁺ nor CD8⁺ T cells directly mediate protection against recurrent *Salmonella* infection

T cells are important mediators of host defense against *Salmonella* infection because mice with targeted defects in CD4⁺ and CD8⁺ T cells show defects in controlling both primary and secondary infection even with attenuated *Salmonella* mutant strains [21–26]. However, given the intricate cross-regulation between T cells and other immune cell subsets, the use of mice with targeted T cell defects cannot discriminate between whether these cells are required for priming protection by other immune mediators or if T cells directly provide protection. To overcome these limitations and determine the specific requirements for CD4⁺ and/or CD8⁺ T cells in protection against recurrent *Salmonella* infection after eradication of primary infection, we compared the susceptibility of antibiotic-treated mice following CD4⁺ and/or CD8⁺ T cell depletion immediately prior to secondary infection. We found CD4⁺ or CD8⁺ T cell depletion either alone or in combination had no significant effect on the level of protection primed by early eradication of primary *Salmonella* infection (Fig. 4A). Since each T cell subset was found to be depleted $\geq 99\%$, these results cannot be attributed to inefficient T cell depletion (Fig. 4B). Taken together, these results demonstrate CD4⁺ and CD8⁺ T cells are non-essential direct mediators of protection against recurrent *Salmonella* infection.

3.3. Protective *Salmonella*-specific antibodies are primed after early infection eradication

Given the sustained protection against recurrent infection even after CD4⁺ and CD8⁺ T cell depletion, and the importance of serum mediated protection especially in mice innately resistance to *Salmonella* infection [27], we also enumerated the impacts of early eradication of primary infection on the anti-*Salmonella* serological response. Remarkably, despite antibiotic treatment beginning day 5, total anti-*Salmonella* IgG in these mice was only modestly reduced compared with mice with persistent infection without antibiotic treatment 45 days after primary infection (Fig 5). Anti-*Salmonella* antibody was sustained for all IgG isotypes, and for IgG2a and IgG2b, the response between antibiotic treated and untreated controls was nearly indistinguishable (Fig 5). By contrast to IgG, anti-*Salmonella* IgM and IgA were each not sustained because the background levels in *Salmonella* infected mice after antibiotic eradication were significantly reduced compared with mice without antibiotic treatment and sustained infection. The anti-*Salmonella* serological IgG response despite early eradication of primary infection was specifically triggered by live bacteria because the same dose of heat-killed *Salmonella* (10^4 bacteria) did not prime a significant increase in antibody titer above the background levels found in naïve control mice (Fig 5).

To determine if *Salmonella*-specific antibody primed after early antibiotic treatment of primary *Salmonella* infection provides protection against recurrent infection, the impact of adoptively transferred serum on infection susceptibility was enumerated. After challenge with a sub-lethal inocula of virulent *Salmonella* (10^4 CFUs), serum from *Salmonella* infected antibiotic-treated mice conferred significant reductions (≥ 10 -fold) in recoverable *Salmonella* CFUs in the spleen and liver 5 days post-challenge, while serum from naïve mice did not significantly impact infection susceptibility (Fig. 6A). Similarly after challenge with a higher dose of virulent *Salmonella* (10^6 CFUs) normally lethal for naïve mice,

protection was partially restored with serum from *Salmonella* infected antibiotic-treated mice while serum from naïve control mice failed to confer protection (Fig. 6B). Together, these results demonstrate *Salmonella*-specific antibody is primed despite early antibiotic-mediated resolution of primary infection, and that adoptive transfer of serum containing anti-*Salmonella* antibody confers protection against recurrent infection.

4. Discussion

Given the widespread use of antimicrobials to eradicate and reduce the long-term complications associated with human typhoid, identifying how this therapy impacts of protection against recurrent infection is an important area for investigation. Although human epidemiological data demonstrating reduced attack rates of recurrent infection in individuals where primary typhoid was treated with antibiotics suggest protective immunity is generated [8,9], these studies are limited by relatively small sample sizes, large heterogeneity in immune responses between individuals, and wide fluctuations in *Salmonella* inocula during natural human infection even during outbreak-type settings. To more definitively address this question, the impact of antibiotic-mediated clearance of primary *Salmonella* infection on protective immunity to recurrent infection was investigated using a murine model of persistent *Salmonella* infection where each of these parameters could be more precisely controlled. We demonstrate that protection is primed against recurrent *Salmonella* infection and sustained equally whether antibiotics are administered during the early (day 5) or later (day 20) phases of primary *Salmonella* infection. These results have important implications for treating and preventing recurrent *Salmonella* infection especially in typhoid endemic areas where re-infection is essentially unavoidable.

Furthermore, using immunological tools and experimental techniques that are more readily performed in rodent models of infection, the mediators of protective immunity primed by antibiotic-mediated eradication of primary *Salmonella* infection were identified. We demonstrate that protection against recurrent infection is largely mediated by *Salmonella*-specific antibody because resistance against secondary infection could be transferred with serum containing high titers of *Salmonella*-specific antibody. These findings are consistent with the demonstration that antibody plays a dominant role in protection against secondary infection in mice containing the resistant allele of *Nramp1* that are inherently resistant to *Salmonella* [27,28], the protection against human typhoid conferred by the Vi polysaccharide vaccine that primes a T cell-independent, *Salmonella*-specific serological response [29–33], and the significantly reduced levels of protection against secondary *Salmonella* infection in B cell (antibody)-deficient mice [12,34,35]. Importantly, since B cells are also potent antigen-presenting cells required for the optimal priming of *Salmonella*-specific Th1 CD4⁺ cells after infection with live-attenuated strains, the lack of protection against recurrent infection in B cell-deficient mice does not discriminate between the antibody-producing and antigen-presenting roles of these cells [34,36]. To clarify these different roles, the ability of serum containing *Salmonella* antibody primed by early eradication of primary infection to transfer protection to naïve mice was demonstrated (Fig. 6). The more modest level of protection achieved with serum transfer compared to intact mice primed by primary *Salmonella* infection with antibiotic eradication (Figs. 1,3 and 6) is most likely due to diminished anti-*Salmonella* antibodies related to adoptive transfer. However, the potential importance of activated macrophages primed by live *Salmonella* that may also contribute to this differences in protection cannot be excluded [28,37]. Nevertheless taken together, these results illustrate the importance of sustained *Salmonella*-specific antibody in protective immunity against recurrent infection.

In sharp contrast to their role in host defense against primary infection, neither CD4⁺ nor CD8⁺ T cells were essential mediators of protective immunity against recurrent infection

because depletion of each cell type individually or in combination did not increase susceptibility to secondary *Salmonella* infection in antibiotic-treated mice [14–16]. Despite these observations, T cells clearly contribute to and play an important role in host defense against *Salmonella* infection because mice with targeted defects in CD4⁺ T cells are highly susceptible to and do not eradicate even attenuated strains of *Salmonella*, and similar defects in host defense against primary *Salmonella* infection are found with T cell depletion [13,16,21–26]. By extension, adoptively transferred T cells primed with attenuated *Salmonella* together with antibody confers protection in naïve mice, while T cell ablation eliminates protection against secondary infection in mice lacking the resistant allele of *Nramp1* [38]. These findings in the mouse model of typhoid are consistent with the clinical observation that recovery from human typhoid and reductions in typhoid-associated complications such as gastrointestinal bleeding or perforation each correlates with the development of cell-mediated immunity [39–41]. Since T cells also play important and critical roles in the maturation and activation of antibody-producing cells, the increased susceptibility in T cell-deficient mice cannot discriminate between whether T cells provide protection indirectly through enhanced and sustained help to antibody-producing cells. Our experiments comparing differences in susceptibility to recurrent infection with T cell ablation immediately prior to secondary infection demonstrate that neither CD4⁺ nor CD8⁺ T cells are essential direct mediators of protection against recurrent *Salmonella* infection.

Protection against recurrent infection demonstrated by reductions in recoverable bacterial CFUs and increased survival despite early antibiotic eradication of primary infection we demonstrate here is consistent with the delayed time to death for antibiotic-treated mice after *S. typhimurium* infection in mice inherently susceptible to virulent *Salmonella* [13]. Although the level of protection primed by antibiotic eradication of primary infection was sharply reduced compared with that primed by an attenuated *Salmonella* mutant that causes more persistent primary infection [13], the level of protection we demonstrate using mice inherently more resistant to virulent *Salmonella* is sharply increased (100% survival in 20 mice) even after challenge with a significantly higher inoculum of virulent *Salmonella* that is normally lethal even in B6.129F1 mice containing a resistant allele of *Nramp1* (Fig. 3). Taken together, these findings suggest vaccination strategies that prime a more robust and sustained serological response compared with primary infection will confer enhanced protection against *Salmonella* infection.

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Fig. 1.

Protection against secondary *Salmonella* infection despite early eradication of primary infection. A. Number of recoverable *Salmonella* CFUs in the spleen and liver day 5 post-secondary challenge (10^4 CFUs) in B6.129F1 mice treated with antibiotics beginning day 20 after primary infection (10^4 CFUs) or control mice treated with antibiotics in parallel without primary infection. B. Number of recoverable *Salmonella* CFUs in the spleen and liver day 5 post-secondary challenge (10^4 CFUs) in B6.129F1 mice treated with antibiotics beginning either day 5 or day 20 after primary infection (10^4 CFUs), or control mice without primary infection. These results are representative of two independent experiments each containing 3–5 mice per group. Bar, one standard error.

Fig. 2.

Enrofloxacin eradicates persistent *S. typhimurium* infection in B6.129F1 mice. Number of recoverable *Salmonella* CFUs in the spleen and liver at the indicated time points after infection for mice with (open squares) or without (filled squares) enrofloxacin (2mg/ml) supplementation in the drinking water beginning day 5 post-infection. These results are representative of two independent experiments each containing 3–5 mice per group per time point. Dotted line, limit of detection.

Fig. 3.

Protection against secondary challenge with virulent *S. typhimurium* despite early eradication of primary infection. Percent survival following secondary challenge with virulent *S. typhimurium* (10^6 CFUs) in mice treated with antibiotics beginning 5 days post-primary infection (10^4 CFUs) (open squares), mice treated with the same dose of heat-killed *Salmonella* (filled circles), or naïve mice given antibiotics in parallel (open circles). The number of mice in each group is indicated and combined from two to three independent experiments.

Fig. 4.

T cell depletion does not impact protection against secondary *Salmonella* infection conferred by antibiotic eradication of primary infection. **A.** Number of recoverable *Salmonella* CFUs in the spleen and liver day 5 post-challenge for mice with CD4⁺ and CD8⁺ T cells depleted beginning one day prior to secondary *Salmonella* challenge (10⁴ CFUs). **B.** Representative FACS plots demonstrating the efficiency of in vivo CD4⁺ and CD8⁺ T cell depletions. The numbers in each plot indicate the percent cells in each gate. These results are combined from two independent experiments containing 7–10 mice per experimental group.

Fig. 5.

Salmonella-specific IgG is sustained despite early eradication of primary *Salmonella* infection. Antibody titers of *Salmonella*-specific IgG, IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3 in the serum of mice 45 days post-infection (10^4 CFUs) treated with enrofloxacin beginning day 5 (open squares) or without antibiotic treatment (filled squares), mice treated the same dose of heat-killed *Salmonella* (filled circles), or naïve mice (open circles). These results represent four independent experiments containing 4–6 mice per group.

Fig. 6.

Adoptively transferred serum from mice eradicated of primary *Salmonella* infection confers protection to naïve recipients. A. Number of recoverable *Salmonella* CFUs in the spleen and liver day 5 after infection with virulent *S. typhimurium* (10^4 CFUs) for mice transferred serum from mice eradicated of primary *Salmonella* or no transfer control mice (top). Number of recoverable *Salmonella* CFUs in the spleen and liver day 5 post-challenge for mice transferred serum from naïve antibiotic-treated mice or no transfer control mice (bottom). B. Percent survival after infection with virulent *S. typhimurium* (10^6 CFUs) in mice transferred serum from mice eradicated of primary *Salmonella* (open squares) or control mice without primary infection (filled squares). These results are representative of two independent experiments containing 8 – 12 mice per group. Bar, one standard error.