Iron Chelation Via Deferoxamine Exacerbates Experimental Salmonellosis Via Inhibition of the Nicotinamide Adenine Dinucleotide Phosphate Oxidase-Dependent Respiratory Burst

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Iron Chelation Via Deferoxamine Exacerbates Experimental Salmonellosis Via Inhibition of the Nicotinamide Adenine Dinucleotide Phosphate Oxidase-Dependent Respiratory Burst

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Competition for cellular iron (Fe) is a vital component of the interaction between host and intracellular pathogen. The host cell requires Fe for the execution of antimicrobial effector mechanisms, whereas most bacteria have an obligate requirement for Fe to sustain growth and intracellular survival. In this study, we show that chelation of host Fe in vivo exacerbates murine salmonellosis, resulting in increased bacterial load and decreased survival times. We further demonstrate that host Fe deprivation results in an inability to induce the NADPH oxidase-dependent production of reactive oxygen, an essential host defense mechanism for the early control of Salmonella typhimurium infection. Thus, altering the equilibrium of intracellular Fe influences the course of infection to the benefit of the pathogen. The Journal of Immunology, 2002, 168: 3458–3463.

Iron (Fe) is an essential factor for many processes in living organisms. During infection, Fe is required by both the host cell and the pathogen. Macrophages require sufficient intracellular Fe to act as a cofactor in the induction of effective antimicrobial defense mechanisms, including the NADPH-dependent oxidative burst and the production of NO catalyzed by inducible NO synthase (iNOS)2 (reviewed in Ref. 1). However, intracellular oxidative burst and the production of NO catalyzed by inducible microbial defense mechanisms, including the NADPH-dependent cellular Fe to act as a cofactor in the induction of effective anti-microbial defense mechanisms, including the NADPH-dependent oxidative burst and the production of NO catalyzed by inducible NO synthase (iNOS) also have an obligate requirement for Fe to support intracellular growth and survival (2). Limiting the availability of intracellular Fe is one way that the host can control the replication of intracellular pathogens, as reflected by the down-regulation of the transferrin receptor in activated macrophages (3). However, the labile equilibrium of Fe availability for both host and pathogen is illustrated by the following two extreme situations: 1) patients suffering from severe anemia show increased susceptibility to salmonellosis (4, 5), and 2) at the other end of the spectrum, patients with either hereditary or dietary Fe overload are at higher risk of developing disease following infection with intracellular bacteria (6). In this study, we report that Fe chelation dramatically exacerbates murine infection with S. typhimurium via inhibition of the host NADPH oxidase-dependent respiratory burst.

Materials and Methods

Mice

C57BL/6 and 129/SvJ mice were bred and housed under specific pathogen-free conditions at the central animal facilities of the Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (Berlin, Germany). Mice of either sex were used at 6–8 wk of age.

Infection with Salmonella enterica var. typhimurium (S. typhimurium)

For all infections, a single colony of S. typhimurium was inoculated into Luria-Bertani (LB) broth (Difco, Detroit, MI) and incubated at 37°C overnight without shaking. Routinely, mice were infected i.p. with 200–600 CFU in 200 μl PBS (Biochrom, Berlin, Germany). For oral infections, mice were starved overnight and infected with 5 × 10^9 bacteria in 200 μl PBS. For each experiment, the input was determined by plating the inoculum on LB agar. Wild-type S. typhimurium as well as the characterized mutant strains SPI2 (7), PhoP (8), and AroA (9) were kindly provided by B. Raupach (Max Planck Institute for Infection Biology, Berlin, Germany). Bacterial load in organs was determined by plating serial dilutions of organ homogenates on LB agar. Organs were weighed before homogenization, and the number of bacteria was calculated per gram of tissue.

Modulation of host Fe

For depletion of Fe in vivo, mice were injected i.p. with 1 mg deferoxamine (Def) or 1 mg lactoferrin (LF; ICN Pharmaceuticals, Aurora, OH) in 100 μl PBS 1 h before infection and again 2 h postinfection. Neither of these compounds was toxic to the mice, as treated but uninfected mice remained healthy.

Effect of Fe-modulating compounds on bacterial growth in culture

Overnight cultures of S. typhimurium were prepared as described above. The OD_{600} was measured, and all cultures were adjusted to OD_{600} 0.1. Def and LF were added at a concentration of 1 mg/ml, and the OD_{600} was measured hourly over an 8-h time period. Triplicate samples were measured, and mean ± SD was calculated.

In vitro killing assay

Bone marrow-derived macrophages (BMMφ) were prepared, as previously described (10), and plated at 1 × 10^5 cells/well in 96-well flat-bottom tissue culture plates in tissue culture medium (DMEM plus 10% FCS, 100 μM L-glutamine, and 100 μM sodium pyruvate; medium and supplements from Biochrom). Where appropriate, cells were activated with 1000 U rIFN-γ for 24 h before infection. S. typhimurium were added at a multiplicity of infection of 5:1, and the plates were centrifuged at 3360 × g for 5 min. After 30-min incubation at 37°C, 5 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO) was added to kill extracellular bacteria and remained in the culture during the course of the assay. At various time points, cells were washed and lysed in 0.1% deoxycholate (Sigma-Aldrich), and the lysate was diluted and plated on LB agar.

Measurement of NO

BMMφ were prepared as detailed for the in vitro killing assay and treated with 1 mg/ml Def or LF. Where appropriate, cells were activated with rIFN-γ (1000 U/ml), LPS (from Escherichia coli; Sigma-Aldrich), or S.
typhimurium (1 × 10⁶/well), or a combination thereof. Supernatants were harvested at 72 h and assayed for nitrite production, as previously described (11).

Measurement of the production of H₂O₂
H₂O₂ was measured as previously described (12). Mice were injected i.p. with 500 µg Na periodate (Sigma-Aldrich), and 4 days later peritoneal cells were harvested, adhered at 1 × 10⁶/well in a 96-well flat-bottom tissue culture plate, and activated overnight with 1000 U/ml rIFN-γ. Def or LF were dissolved in assay medium (Earle’s balanced salt solution containing 0.56 mM Phenol Red and 20 U/ml HRP; all components from Sigma-Aldrich) and added to the cells along with 2 µg/ml PMA (Sigma-Aldrich). The reaction from one set of cells was immediately stopped by the addition of 10 µl 1 N NaOH, and this represented t = 0. At various time points after stimulation, the reaction was stopped and the OD was measured at 600 nm. A positive control of a titration of H₂O₂ was included on each plate.

Results
Chelation of host Fe by Def exacerbates infection with S. typhimurium
We first examined whether the in vivo modulation of host Fe content altered the course of Salmonella infection. Prior treatment of C57BL/6 mice with LF slightly exacerbated infection with S. typhimurium at 4 h postinfection, but by 24 h there was no significant difference in bacterial loads in livers and spleens when compared with untreated mice (Fig. 1). In contrast, administration of the Fe chelator, Def, before infection, slightly increased the bacterial load in both liver and spleen of treated mice by 4 h postinfection, and resulted in a 2- to 3-log increase after 24 h. In contrast to LF, which binds only extracellular Fe, Def is a global Fe chelator that binds both intra- and extracellular Fe. The effectiveness of the Def treatment regime was confirmed by a 20–30% reduction in Fe in the serum of the animals (data not shown), which is within the range previously reported to be effective for Fe deprivation following dietary manipulation (13).

It has been shown that the Nramp 1 protein plays an important role in resistance to murine Salmonella infections (14). This protein has high homology to Nramp 2, which functions as a divalent metal ion transporter and has been linked to the human hereditary Fe overload condition, hemachromatosis (15). We therefore compared the effects of Fe chelation in 129/SvJ mice (129), which carry the resistant Nramp 1 allele, vs susceptible C57BL/6 mice (B6). As expected, the bacterial loads in 129 mice in the absence of Def were lower than those of the B6 mice; however, Def treatment substantially exacerbated infection in both mouse strains, resulting in an ~4-log increase in bacterial burden in spleen (Fig. 2A) and liver (data not shown) by 48 h postinfection. Furthermore, Def treatment greatly reduced the survival times of resistant mice, resulting in the death of all mice by day 4, while the untreated 129 mice survived the infection for at least 11 days (Fig. 2B). Thus, functional Nramp 1 did not overcome the impairment in host bacterial effector mechanisms induced by cellular Fe deprivation. Moreover, Def does not globally disrupt host defense mechanisms, as identical experiments using Listeria monocytogenes revealed no differences in either survival or bacterial loads between control and Fe-depleted mice over a 7-day time period (data not shown).

Def treatment inhibits the NADPH-dependent oxidative burst in vitro
To elucidate the exact mechanisms by which Fe deprivation exacerbated Salmonella infection, we examined the ability of macrophages treated with Def or LF to kill intracellular Salmonella. In the absence of IFN-γ activation, untreated macrophages or those pretreated with LF were able to restrict the intracellular growth of the bacteria over a 4-h time period (Fig. 3A). In contrast to this, bacterial replication was unrestrained in macrophages treated with Def. Prior activation of macrophages with IFN-γ enhanced the killing efficiency for Salmonellae, but consistently the Def-treated

![FIGURE 1](http://www.jimmunol.org/)  Def treatment exacerbates S. typhimurium infection. Mice were pretreated with Def or LF and infected with S. typhimurium. Bacterial loads in spleens and livers were determined at 4 and 24 h postinfection. Each symbol represents an individual mouse. Results shown are representative of at least four separate experiments. *, Value of p < 0.05 (Mann-Whitney test).

![FIGURE 2](http://www.jimmunol.org/)  A: C57BL/6 vs 129. Bacterial loads were determined in spleens at day 2 postinfection. Each symbol represents an individual mouse. *, Value of p < 0.05 (Mann-Whitney test). B: Survival times were monitored in Nramp 1-susceptible (C57BL/6) and -resistant (129) mice (five mice per group).
cells were less efficient (Fig. 3B). The two major microbicidal effector mechanisms of macrophages are the production of NO via the induction of iNOS, and the production of oxygen radicals by the NADPH oxidase-dependent respiratory burst. The latter process represents the primary anti-Salmonella mechanism within the early phase of infection, whereas NO plays a role later in disease (16, 17). We therefore determined whether these pathways were influenced by Fe depletion. The production of NO as an indicator of the iNOS-dependent pathway was measured in vitro. Both the production of NO by macrophages primed with IFN-γ, as well as those primed with IFN-γ and infected with Salmonella were unaffected by prior treatment of the macrophages with Def or LF (Fig. 3C). Additionally, the amount of nitrite measured in the serum of Salmonella-infected mice did not differ between Fe-depleted and control mice (data not shown). To determine whether the generation of oxygen radicals was affected by Def or LF, we tested whether macrophages could produce H₂O₂ in response to PMA stimulation in the presence of these compounds. Because the optimal conditions for generating a respiratory burst are in vivo priming followed by in vitro stimulation, as previously described we used peritoneal macrophages rather than BMMø (17). The presence of Def inhibited the PMA-induced production of H₂O₂ in comparison with control macrophages (Fig. 3D). Conversely, the presence of LF significantly enhanced H₂O₂ production. These results suggest that even under optimal conditions of priming and subsequent activation, Def-mediated Fe chelation significantly inhibits the production of oxygen radicals as measured by H₂O₂ production. Thus, we propose that the NADPH-dependent respiratory burst, rather than the iNOS pathway, is inhibited by Fe chelation, providing a possible explanation for the increased bacterial loads in Fe-deprived mice early after infection.

Fe chelation permits the growth in vivo of the SPI2 mutant of S. typhimurium

To verify these in vitro observations in vivo, several well-characterized mutants of S. typhimurium were used. The SPI2 strain has mutated genes of the Salmonella pathogenicity island 2, and is attenuated in wild-type mice (7). This strain of Salmonella grows unimpeded in mice deficient for gp91phox, a component of the NADPH oxidase complex, who are unable to mount a productive respiratory burst (18). We reasoned that if Def inhibited the induction of the NADPH oxidase-dependent respiratory burst, then the SPI2 mutant would replicate in Def-treated mice. Indeed, by 48 h post-i.p. infection, there was significant growth of the SPI2 mutant in the livers and spleens of Def-treated mice (Fig. 4), whereas already at this time point the untreated mice were beginning to control the infection. In contrast, the AroA mutant, which is metabolically attenuated due to a deficiency in aromatic amine synthesis, was unable to replicate in either control or Fe-depleted animals.

To test the possibility that the exacerbation of infection is a direct result of the capability of Salmonella to use Def-bound Fe to enhance growth (19), the effect of Def and LF was tested on Salmonella growth in vitro. The addition of either compound did not affect the in vitro growth of the two Salmonella strains investigated.

This content is from the article titled "Fe DEPRIVATION AND SALMONELLOSIS". The article discusses the effects of iron deprivation on the immune response and bacterial growth, particularly focusing on the role of iron in respiratory burst generation and its impact on Salmonella infections. The text highlights the importance of iron in the immune response and suggests mechanisms by which iron deprivation can exacerbate Salmonella infections. The article also presents experimental evidence supporting these hypotheses, including in vitro and in vivo studies.
either in broth cultures (Fig. 5) or in tissue culture medium (data not shown). Thus, these data suggest that modulation of host Fe via the addition of Fe-binding molecules significantly alters the ability to control infections with Salmonella. Notably, chelation of Fe by Def appears to affect critical antibacterial mechanisms of the host early in infection.

We further extended these observations to consider whether systemic Fe chelation altered the course of Salmonella infection when the bacteria were administered via the natural route of infection, i.e., per os. At days 2 (data not shown) and 4 postinfection, bacterial loads in spleens and mesenteric lymph nodes were significantly higher in Fe-deprived mice infected with either wild-type or SPI2 Salmonella strains. Indeed, untreated mice controlled the infection with SPI2 to an extent that bacteria were undetectable in the lymph nodes and 1 log lower in the spleen (Fig. 6). In contrast, mice treated with Def were unable to control SPI2 infection, and organisms persisted in both organs. As the AroA strain used in the previous experiment is nonreplicating in vivo and therefore is not a good readout for the host immune response, we included the PhoP mutant in oral infection experiments. This mutant is a good readout for the host immune response, as the previous experiment is nonreplicating in vivo and therefore is not

Discussion

The availability of intracellular Fe must remain in a delicate balance between intracellular pathogens and their host cell. On the one hand, sufficient Fe must be available for the induction of antimicrobial mechanisms in host cells, while, on the other hand, the restriction of intracellular Fe can prevent the growth of the bacteria within the cell. In this study, we describe the exacerbation of murine salmonellosis following the modulation of this balance via global Fe chelation with Def. This compound enters the cell, thereby depleting both extracellular and intracellular Fe stores, and is used clinically to treat a variety of diseases resulting from Fe overload, such as hemachromatosis. Patients suffering from these conditions also show increased susceptibility to infections with intracellular bacteria (5, 6). Treatment with Def resulted in a 3-log increase in bacterial burden compared with untreated mice, whereas LF-treated mice controlled infection slightly better by 24 h and certainly did not exacerbate infection. Thus, two different Fe-binding molecules had opposite effects on the outcome of infection with S. typhimurium. Recently, the antimicrobial activity of LF has been ascribed to the action of a peptide contained within the bovine LF molecule (20). However, both in vivo and in vitro, this peptide had no effect on the growth of Salmonella (data not shown), and LF has no direct influence on S. typhimurium growth in broth culture. Therefore, we propose that LF does not exacerbate Salmonella infection, as it binds only extracellular Fe and leaves the host intracellular Fe stores intact. Def, in contrast, can bind extracellular and intracellular Fe, depriving both host and bacteria of Fe, a situation that early in the infection has greater impact on the host.

The Nramp 1 gene has previously been shown to confer resistance to Salmonella in the early phase of infection. Cloning and
sequence analysis of this protein revealed a transmembrane protein with high homology to the metal ion transporter Nramp 2. Susceptibility to Salmonella infections is associated with a glycine to aspartate substitution at position 169 of the fourth transmembrane domain of Nramp 1. In studies using transfected macrophages, Fe was transported more efficiently into latex bead phagosomes of resistant macrophages than those of susceptible cells (21). Furthermore, the addition of Fe to Nramp 1-susceptible macrophages stimulated the growth of the intracellular bacterium Mycobacterium avium, whereas similar treatment of Nramp 1-resistant cells increased their antimicrobial capacity, although within a very narrow dose range (22). More recently, using Xenopus oocytes, it has been unequivocally demonstrated that Nramp 1 functions as a pH-dependent transporter of metal ions including Fe$^{3+}$ (23). Despite this, a resistant Nramp 1 allele could not overcome the effects of Def, suggesting that this treatment has removed all available transportable Fe.

The host requirements for the control of Salmonella infections have been studied extensively. Using a combination of knockout mice and mutant Salmonella strains, it was recently revealed that the control of murine infection with S. typhimurium critically depends upon the NADPH-dependent generation of reactive oxygen intermediates as well as the iNOS-catalyzed NO production. Both in vitro and in vivo studies revealed distinct kinetics of the induction of these two macrophage antimicrobial mechanisms. In vivo, iNOS was required between days 4 and 7 postinfection, whereas mice lacking a component of the NADPH oxidase succumbed to Salmonella within 2 days of the infection (16). Similarly, in vitro analyses revealed that macrophage killing of S. typhimurium within the first few hours after phagocytosis coincided with superoxide anion and hydrogen peroxide production (17). In accordance with this, patients suffering from chronic granulomatous disease or defective priming of the oxidative burst are highly susceptible to recurrent, disseminated salmonellosis (24, 25). Confirmation of the impact of Def treatment on the induction of the oxidative burst was provided by the unrestrained growth of the SPI2 mutant in Def-treated animals, despite its attenuation in control mice. This mutant Salmonella strain has previously been shown to grow only in mice deficient in gp96$^{\text{trans}}$, a critical component of the NADPH oxidase (7, 18). In contrast to this, the growth of a PhoP mutant of Salmonella in mice is unaffected by Def treatment, suggesting that it is an impairment of the NADPH-dependent oxidative burst, which is required to control SPI2 growth, rather than a general ability of Salmonella to use Def-bound Fe for their intracellular replication.

The data presented in this study further underline the contribution of the respiratory burst to the early control of Salmonella infections and clearly demonstrate the critical role of host Fe to the induction of this microbicidal mechanism. The most obvious explanation of how Def reduces the respiratory burst is via the chelation of available Fe within the macrophage, thereby preventing production of hydroxyl radicals via the Fe-catalyzed Haber-Weiss reaction (6). These radical species are assumed to be responsible for the damaging effects of Fe in overload conditions, and are presumably responsible for the inhibition of the growth of Salmonella in normal mice. As macrophages lack myeloperoxidase, which is required for a second pathway of hydroxyl radical generation, inhibition of the Haber-Weiss reaction would significantly reduce the ability of these cells to produce antibacterial effector molecules.

Previous reports have provided contradictory evidence concerning the role of Fe deficiency in Salmonella infection. In accordance with results presented in this study, mice injected with 2,3-dihydroxybenzoic acid, a phenolic Fe chelator, were more susceptible to infection with wild-type S. typhimurium, whereas a mutant Salmonella strain unable to synthesize its own Fe uptake molecule was unaffected by the treatment (26). In contrast to these findings, mice whose available Fe pool was reduced by dietary manipulation survived Salmonella infection better than mice receiving normal food (27). The apparent discrepancy in these results may reflect the contrasting availability of Fe absorbed via the intestine compared with the systemic administration of Fe-chelating compounds. Interestingly, although these studies used reduction in dietary Fe to modulate host Fe content, the mice were not challenged orally. We have demonstrated in this study that the chelation of host Fe not only affected systemic Salmonella infection, but also increased dissemination of the bacteria from the intestine following infection via the natural route.

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**References**


