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Macrophage Permissiveness for *Legionella pneumophila* Growth Modulated by Iron

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We have investigated the modulation of iron in two populations of macrophages which differ in susceptibility to *Legionella pneumophila* intracellular proliferation. Previously, we reported that thioglycolate-elicited peritoneal macrophages obtained from the inbred A/J mouse strain readily support the intracellular growth of *L. pneumophila*, while resident macrophages from the same strain do not. In this study, we show that A/J elicited macrophages exhibit markedly higher expression of transferrin receptor and intracellular iron content than A/J resident macrophages. Furthermore, apotransferrin and desferrioxamine inhibited the intracellular proliferation of *L. pneumophila* in elicited macrophages, and this suppression was reversed by the additions of Fe-transferrin or ferric nitrilotriacetate. Fe-transferrin and ferric nitrilotriacetate did not further increase the intracellular proliferation of *L. pneumophila* in thioglycolate-elicited macrophages. However, ferric citrate and ferric nitrilotriacetate stimulated in a dose-dependent manner the growth of *L. pneumophila* in resident macrophages. Furthermore, equimolar concentrations of desferrioxamine reversed the stimulatory effect of iron in these resident cells. These data provide evidence supporting the hypothesis that differences in susceptibility to *L. pneumophila* growth between permissive elicited macrophages and nonpermissive resident macrophages from the A/J mouse strain are due to intracellular availability of iron.

Legionella pneumophila, the etiological agent of legionellosis in humans, is a facultative intracellular pathogen that can successfully evade host defenses by parasitizing and multiplying within mononuclear phagocytes (10, 15, 16). Early studies on the nutritional requirements of *L. pneumophila* reported a necessity for relatively large amounts of iron for optimum growth in vitro (12, 27, 30). Several laboratories also reported that the extracellular and intracellular growth of *L. pneumophila* are significantly inhibited by different iron chelators as well as by lactoferrin and serum transferrin (Tf) (3–5, 25, 26). In addition, Byrd and Horwitz showed that gamma interferon restricts the growth of *L. pneumophila* in human monocytes by reducing the intracellular iron availability by down-regulation of ferritin content and Tf receptor (TfR) expression (4–6).

Although guinea pig and human macrophages are permissive for *L. pneumophila*, macrophages from most mouse strains are known to restrict the growth of this organism, even at high infectivity ratios (35–37). Previously, we reported that thioglycolate-elicited peritoneal macrophages obtained from the inbred A/J mouse strain readily support the intracellular growth of *L. pneumophila* (36). However, in contrast to elicited macrophages, resident macrophages from A/J mice did not support the growth of this bacterium (36). In the present study, we examined the possible involvement of iron in the susceptibility of permissive elicited versus nonpermissive resident macrophages from the A/J mouse strain for *Legionella* growth.

MATERIALS AND METHODS

Reagents. All chemicals and media were obtained from the Sigma Co. (St. Louis, Mo.) unless otherwise noted. Human Fe-Tf corresponded to the low-endotoxin lot. Ferric citrate was prepared by mixing in a 1:1 ratio trisodium citrate with ferric chloride and then adjusting pH to neutrality with 1 N NaOH. Ferric nitrilotriacetate (FeNTA) was prepared in the same way by mixing disodium nitrilotriacetate with ferric chloride as previously described (5). Rat immunoglobulin G2 monoclonal antibodies against TfR were purchased from Pharmingen (San Diego, Calif.), and rat immunoglobulin G2 monoclonal antibodies against MAC1 (CD11b) were obtained from Boehringer Mannheim (Indianapolis, Ind.).

Mice. Inbred female A/J mice were purchased from Jackson Laboratory, Bar Harbor, Maine, at approximately 6 weeks of age. They were kept in groups of 8 to 10 and fed commercial mouse chow and water ad libitum.

Bacteria. A virulent strain of *L. pneumophila*, serogroup 1, was obtained initially from a case of legionellosis at Tampa General Hospital. The bacteria were maintained frozen at −70°C and, prior to use, grown on buffered charcoal yeast extract agar (Difco Laboratories, Detroit, Mich.) for 48 h.

Macrophages. Resident or elicited macrophages, obtained by intraperitoneal injection of 3 ml of thioglycolate medium (Difco) 4 days prior to harvesting, were collected by peritoneal lavage using 5 ml of phosphate-buffered saline (PBS) with 2% fetal calf serum (HyClone, Logan, Utah). Cells were washed in PBS and resuspended in RPMI 1640 plus 10% fetal calf serum. Macrophage survival was determined by trypan blue exclusion. In all cases, viability was greater than 95%.

***L. pneumophila* CFU assay.** Macrophages (10^5 cells per well) were made to adhere to 96-well tissue culture plates (Costar, Cambridge, Mass.) for 2 h at 37°C to remove the nonadherent cells. After the monolayer was washed, cells were preincubated with different iron sources or iron chelators for 24 h. Macrophages were then infected with *L. pneumophila* (10^6 bacteria per well) for 30 min at 37°C, and the nonphagocytosed *L. pneumophila* was removed by washing 3 times with Hanks' balanced salt solution. The cells were incubated for either 0,

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TABLE 1. Fluorescence-activated cell sorter analysis of permissive and nonpermissive macrophages[a]

Macrophage population	% Cells positive for:		
	MAC1	TfR	TfR and MAC1[b]
Resident	85.9 ± 11.4	8.0 ± 1.3	7.0 ± 2.0
Elicited	91.2 ± 6.8	78.3 ± 17.2	71.3 ± 16.1

[a] The indicated macrophages were combined with fluorescein isothiocyanate-labeled anti-MAC1 (CD11b) and R-phycoerythrin-labeled anti-TfR for 30 min at 4°C. The stained cells were analyzed with a FACScan flow cytometer, using two-color analysis. The data are the means ± SD for three experiments.

[b] Percent of double-positive cells. The difference for TfR between the macrophage populations is statistically significant ($P < 0.001$).

24, or 48 h with fresh medium containing the same additions as the pretreatment. At the end of the incubation period, the monolayers were washed and lysed with 100 μl of 0.1% saponin per well. The resulting lysates were diluted in Hanks' balanced salt solution and plated on buffered charcoal yeast extract agar to determine the CFU as described previously (36).

Iron determination. The iron content of peritoneal macrophages was determined by a modification of Carter's method (7). Briefly, macrophages (10^7 cells) were made to adhere to a 25-cm^2 flask (Costar) and incubated for 24 h. The supernatants were removed, and monolayers were washed five times with saline. The cell-associated iron content was determined by lysing the cell monolayer in 1 ml of water and then adding an equal volume of 6 N HNO_3. Samples were heated at 90°C in a heating block overnight to release protein-associated iron. All iron was reduced to Fe^{2+} with 3% thioglycolic acid. The pH was raised to 5.0 with NH_4OH, and the sample was added to a solution of 0.3% ferrozine in 2 M acetate buffer (pH 5.0). Ferrous iron was then quantitated spectrophotometrically at 562 nm. The concentration of iron was calculated by comparison with a standard solution of iron.

TfR expression. Resident or elicited macrophages (10^6 cells per tube) were combined with fluorescein isothiocyanate-labeled anti-MAC1 (Pharmingen) and R-phycoerythrin-labeled anti-TfR (Pharmingen) for 30 min at 4°C. The cells were washed with cold PBS and fixed with 1% paraformaldehyde-PBS. The stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.), by using two-color analysis for fluorescein isothiocyanate- and phycoerythrin-positive cells in the gated macrophage population.

Analysis of results. The Student *t* test or analysis of variance was used to determine the statistical probabilities.

RESULTS

The level of TfR expression was evaluated in elicited and resident macrophages. The results of flow cytofluorometric analysis for each macrophage population are shown in Table 1. Resident macrophages were only 8.0% positive for TfR, while elicited macrophages were 78.3% positive. Since one of the major functions of Tf is the transport and delivery of iron to cells, the cellular iron content was determined for both macrophage populations. As would be expected, the higher TfR expression correlated with a greater iron content. The iron content of elicited macrophages was increased 2.8-fold over that of resident macrophages (21.0 versus 7.5 ng/10^6 cells, respectively).

To test whether this rise in iron entrance through the receptor could account for the increase in intracellular growth of *L. pneumophila*, elicited macrophages were incubated with anti-TfR antibodies, infected with *L. pneumophila*, and incubated for 24 and 48 h. As is shown in Fig. 1, blocking the binding of Tf to its receptor induced a decrease in *L. pneumophila* growth inside the macrophages. A nonspecific effect of anti-TfR antibody was ruled out since an equal concentration of an irrelevant antibody (anti-MAC1) did not cause any decrease in *L. pneumophila* intracellular multiplication (Fig. 1).

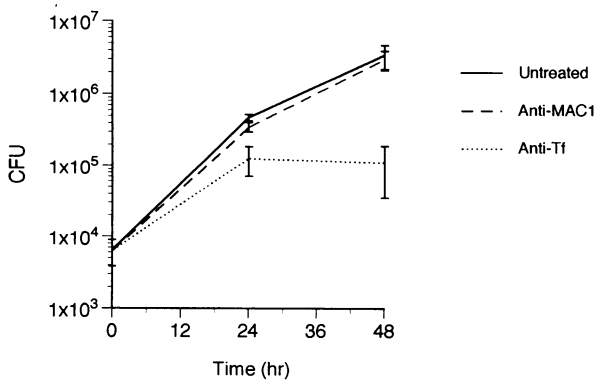

FIG. 1. Anti-TfR effects on intracellular proliferation of *L. pneumophila* in permissive macrophages. Elicited macrophages from A/J strain mice were infected with *L. pneumophila* and incubated with anti-TfR (0.1 mg/ml), anti-MAC1 (0.1 mg/ml), or control medium. CFU were determined at 0, 24, and 48 h. The data are the means ± standard deviation (SD) of three experiments.

To further test iron involvement, the effect of Tf and apotransferrin (apoTf), the iron-free protein, on the replication of *L. pneumophila* in elicited macrophages was examined. apoTf inhibited the proliferation of *L. pneumophila* in a dose-dependent manner (Fig. 2). This effect was reversed by Tf, suggesting that both molecules interact with the same receptor and, as a consequence, blocking of Tf uptake can inhibit the intracellular growth of *L. pneumophila*.

Since the siderophore desferrioxamine (DFO) can penetrate inside the cell and chelate equimolar amounts of ferric iron ($K_a = 10^{31}$) from the so-called iron transient pool in the cytoplasm (11, 19, 20, 31, 34), the replication of *L. pneumophila* in elicited

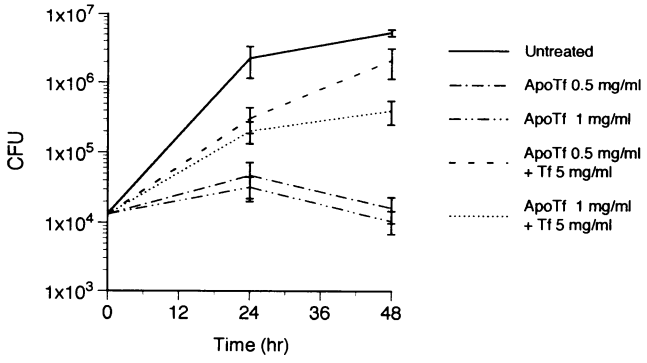

FIG. 2. apoTf effects on the intracellular proliferation of *L. pneumophila* in permissive macrophages. Elicited macrophages from A/J strain mice were infected with *L. pneumophila* and incubated with apoTf and Tf as indicated in the text. CFU were determined at 0, 24, and 48 h. The data are the means ± SD of three experiments.

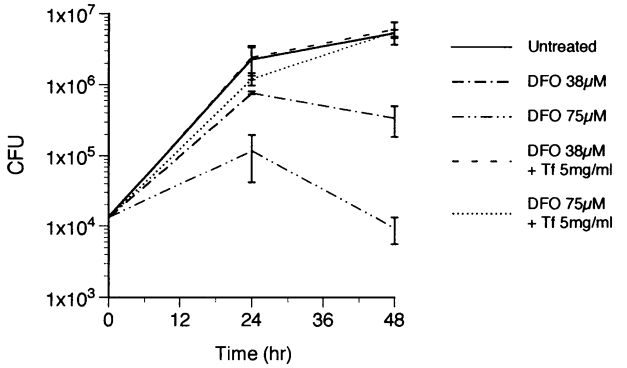

FIG. 3. Tf restores the inhibition induced by DFO in permissive macrophages. Elicited macrophages from A/J strain mice were infected with *L. pneumophila* and incubated with DFO and Tf as indicated in the text. CFU were determined at 0, 24, and 48 h. The data are the means ± SD of three experiments.

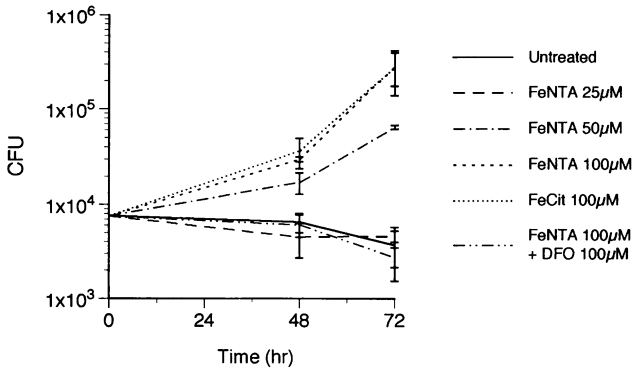

FIG. 5. FeNTA or Tf does not stimulate the intracellular proliferation of *L. pneumophila* in permissive macrophages. Elicited macrophages from A/J strain mice were infected with *L. pneumophila* and incubated with FeNTA and Tf as indicated in the text. CFU were determined at 0, 24, and 48 h. The data are the means ± SD of three experiments.

macrophages was studied in the presence of different concentrations of this compound. When compared with the replication of *L. pneumophila* in untreated elicited macrophages, DFO reduced the rate of bacterial replication in a dose-dependent fashion by as much as fourfold (Fig. 3). This inhibitory effect was not due to an effect on the viability of macrophages, as determined by trypan blue exclusion (data not shown). Moreover, Tf completely restored the capacity of DFO to inhibit *L. pneumophila* multiplication (Fig. 3). The iron salt FeNTA was also able to reverse this inhibitory effect, indicating that the ability of DFO to chelate iron is responsible for the blockade of growth (Fig. 4). The addition of 5 mg of Tf per ml or 100 μM FeNTA did not further increase the intracellular proliferation of *L. pneumophila* in elicited macrophages (data not shown).

To examine whether resident macrophages restrict *L. pneumophila* growth because of reduced intracellular availability of iron, different concentrations of iron salts were added to the cultures. FeNTA stimulated the intracellular proliferation of *L. pneumophila* in a dose-dependent manner, becoming statistically significant at 50 μM or greater (Fig. 5). Ferric citrate showed a similar effect (Fig. 5). Furthermore, the increased intracellular growth induced by iron was completely reversed by adding equimolar concentrations of DFO (Fig. 5).

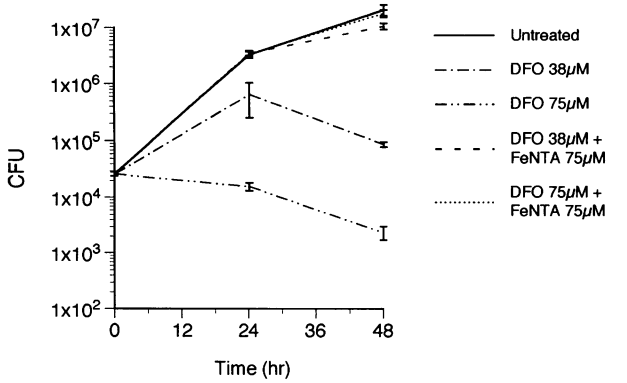

FIG. 4. The ferric salt FeNTA restores the inhibition induced by DFO in permissive macrophages. Elicited macrophages from A/J strain mice were infected with *L. pneumophila* and incubated with DFO and FeNTA as indicated in the text. CFU were determined at 0, 24, and 48 h. The data are the means ± SD of three experiments.

DISCUSSION

Our study demonstrates that differences in permissiveness to *L. pneumophila* growth between elicited macrophages and resident macrophages from A/J mice are due, at least in part, to intracellular iron availability. Elicited A/J macrophages exhibited high levels of TfR expression (78.3%) compared with resident A/J macrophages (8.0%). Hamilton et al. (14) showed that murine thioglycolate-elicited macrophages exhibit a greater number of TfR (a total of 18,600 sites per cell) than resident cells (2,700 sites per cell). They found that this modulation does not appear to result from differential shifts between surface and internal loci. Since one of the major functions of Tf is the transport and delivery of iron to cells, one might expect an increase in the uptake of Tf in elicited macrophages and, as a consequence, greater incorporation of iron into the cells. Using a modification of Carter's colorimetric method, we detected in elicited macrophages 2.8-fold more iron than in resident macrophages. Data similar in terms of differences in cellular iron content between resident and thioglycolate-elicited macrophages were reported by Alford et al. (1). Birgegard and Caro also demonstrated that thioglycolate mouse macrophages incorporated more iron from ^{59}Fe-Tf than did resident macrophages (2). They also found that the synthesis of ferritin was increased in thioglycolate-elicited macrophages and that the ^{59}Fe incorporated into the cell was associated with this iron storage protein. Furthermore, Esparza and Brock reported that the release of iron occurred more rapidly from resident macrophages than from thioglycolate-stimulated macrophages after loading the cells with ^{59}Fe-Tf–anti-Tf immune complexes (11). Therefore, the higher iron content found in elicited macrophages could be the result of an increase in TfR, in ferritin synthesis, and/or in iron uptake as well as of a decrease in the release of iron from the cells.

Iron uptake in most mammalian cells seems to be mediated mainly by the TfR. The interaction between Tf and cells involves several steps (9, 23). The Tf binds to its membrane receptor. The Tf-TfR complexes accumulate in clathrin-coated pits and internalize as clathrin-coated vesicles. The vesicles

lose their clathrin coats in an ATP-dependent process and form smooth-surfaced vesicles or endosomes. These endosomes then fuse with an uncoupling vesicle, reducing the internal pH to 5.0 and causing the dissociation of Tf from its receptor. The free receptor congregates in one membrane section of the uncoupling vesicle and buds off to be recycled back to the plasma membrane. The Fe^{3+} released inside the acidic endosome translocates into the *trans* side of the vesicular membrane through a process that requires reduction of Fe^{3+} to Fe^{2+} and is presumably mediated by a membrane oxidoreductase. In the cytosol, the reduced iron is delivered to the so-called low-molecular-weight iron pool or chelatable-iron pool and is subsequently delivered to ferritin for storage or to other sites for metabolism.

In this study, we inhibited the intracellular proliferation of *L. pneumophila* in elicited macrophages by directly chelating iron from this labile pool. This effect was reversed when Fe-Tf was added to the culture. Since DFO does not directly remove significant amounts of iron from Tf (24), the chelation of iron from this cytoplasmic pool was probably replaced by the iron released and translocated from the Tf-TfR complex located inside the acidic vacuoles of macrophages (31). Thus, blocking the uptake of iron at earlier steps also induced restriction on the intracellular growth of *L. pneumophila*, since antibodies against TfR or apoTf suppressed the intracellular growth of *L. pneumophila.*

Early studies on the nutritional requirements of *L. pneumophila* showed that *L. pneumophila* requires relatively large amounts of iron for optimum growth in vitro (12, 27, 30). In addition, the extracellular growth of *L. pneumophila* is significantly inhibited by different iron chelators, as well as by lactoferrin and serum transferrin (3, 25, 26). More recently, Byrd and Horwitz showed that gamma interferon, DFO, and lactoferrin restricted the multiplication of *L. pneumophila* in human monocytes by reducing the intracellular iron availability (4–6). Here, we have shown that the proliferation of *L. pneumophila* in elicited peritoneal macrophages of the A/J strain is dependent on the intracellular availability of iron. Our data also demonstrate that normally nonpermissive resident macrophages can become permissive in the presence of iron salts. Both ferric citrate and FeNTA stimulated the growth of *L. pneumophila* in the macrophages, and this stimulation was reversible by DFO. In addition, while iron promoted the growth of *L. pneumophila* in resident macrophages, neither FeNTA nor Tf further stimulated the intracellular proliferation of *L. pneumophila* in elicited macrophages, indicating that the latter population does not express any iron restriction.

Although resident macrophages express very low levels of TfR, these cells like most mammalian cells are able to incorporate in vitro iron chelators such as ferric citrate or FeNTA independent of the TfR-mediated uptake system (21, 29, 32, 34). The mechanism of incorporation is not clear but seems to be mediated either by fluid endocytosis or through a Tf-independent pathway which might involve the expression of cryptic transporters capable of binding these iron-organic anion chelators (32). Using the Prussian blue assay, we have found that peritoneal macrophages can incorporate ferric salts into the cytoplasm (data not shown). In fact, MacDonald et al. have shown that the rate of iron uptake by macrophages is 10 to 300 times greater from iron salts than from Tf (21). Thus, although the exact pathway of iron entrance is not known, our data show that *L. pneumophila* is capable of using this cytoplasmic iron for intracellular replication.

The mechanism by which *L. pneumophila* acquires iron is not completely understood. However, Johnson et al. (17) have shown that the uptake of iron by *L. pneumophila* requires a NADH-dependent iron reductase enzyme which is associated with the periplasmic fraction. Additionally, several studies have shown that mobilization of iron from ferritin requires reducing conditions involving NADH (8, 18, 33). Whether *L. pneumophila* produces siderophores is uncertain (13, 28). Thus, whether *L. pneumophila* takes up iron either from the labile iron pool by production of siderophores, from the ferritin molecule by an NADH-dependent process, or from any other unknown mechanism remains to be determined. However, while the mechanism by which *L. pneumophila* takes up iron is undetermined, our data and data of others indicate that the presence of iron is important for growth of *L. pneumophila*. Furthermore, this iron requirement is at least one reason why *L. pneumophila* grows in thioglycolate-elicited macrophages from A/J mice but not in resident macrophages.

The mechanism by which macrophages from other mouse strains restrict the intracellular growth of *L. pneumophila* remains to be determined. We do not believe that iron may explain such differences. Preliminary experiments have shown that the levels of TfR and cellular iron content between permissive A/J and nonpermissive BALB/c elicited macrophages were similar. Additionally, Alford et al. (1) obtained a similar iron level in nonpermissive BDF1 thioglycolate macrophages. Furthermore, neither iron salts nor Tf could stimulate the intracellular growth of *L. pneumophila* in nonpermissive BALB/c elicited macrophages (unpublished data). Experiments are currently in progress in order to elucidate the mechanisms involved in *L. pneumophila* permissiveness among macrophages from different mouse strains.

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