

# Lactoferrin Inhibits or Promotes *Legionella pneumophila* Intracellular Multiplication in Nonactivated and Interferon Gamma-activated Human Monocytes Depending upon Its Degree of Iron Saturation

## Iron-Lactoferrin and Nonphysiologic Iron Chelates Reverse Monocyte Activation Against *Legionella pneumophila*

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### Abstract

We have been exploring the role of iron in the pathogenesis of the intracellular bacterial pathogen *Legionella pneumophila*. In previous studies, we have demonstrated that *L. pneumophila* intracellular multiplication in human monocytes is iron dependent and that IFN $\gamma$ -activated monocytes inhibit *L. pneumophila* intracellular multiplication by limiting the availability of iron. In this study, we have investigated the effect on *L. pneumophila* intracellular multiplication of lactoferrin, an iron-binding protein which is internalized via specific receptors on monocytes, and of nonphysiologic iron chelates which enter monocytes by a receptor-independent route.

Apolactoferrin completely inhibited *L. pneumophila* multiplication in nonactivated monocytes, and enhanced the capacity of IFN $\gamma$ -activated monocytes to inhibit *L. pneumophila* intracellular multiplication. In contrast, iron-saturated lactoferrin had no effect on the already rapid rate of *L. pneumophila* multiplication in nonactivated monocytes. Moreover, it reversed the capacity of activated monocytes to inhibit *L. pneumophila* intracellular multiplication, demonstrating that *L. pneumophila* can utilize iron from the lactoferrin-lactoferrin receptor pathway. The capacity of iron-lactoferrin to reverse monocyte activation was dependent upon its percent iron saturation and not just its total iron content. Similarly, the nonphysiologic iron chelates ferric nitrilotriacetate and ferric ammonium citrate completely reversed and ferric pyrophosphate partially reversed the capacity of IFN $\gamma$ -activated monocytes to inhibit *L. pneumophila* intracellular multiplication, demonstrating that *L. pneumophila* can utilize iron derived from nonphysiologic iron chelates internalized by monocytes independently of the transferrin and lactoferrin endocytic pathways.

This study suggests that at sites of inflammation, lactoferrin may inhibit or promote *L. pneumophila* intracellular multiplication in mononuclear phagocytes depending upon its degree of iron saturation. In addition, this study suggests a

potential role for PMN in host defense against *L. pneumophila*—providing apolactoferrin to infected monocytes—and it supports the concept that PMN and monocytes may cooperate in host defense against intracellular parasites and other pathogens. (*J. Clin. Invest.* 1991. 88:1103–1112.) Key words: iron nitrilotriacetate • transferrin • transferrin receptor • ferritin • ferric ammonium citrate • polymorphonuclear leukocytes

### Introduction

Iron plays an important role in the energy metabolism of microorganisms including those pathogenic for humans. In the case of extracellular bacterial pathogens, iron has been shown to enhance virulence (1). In the human body, iron is relatively unavailable extracellularly as it is bound to the high-affinity iron-binding proteins transferrin and lactoferrin. To obtain iron, extracellular bacteria have evolved mechanisms, such as siderophores and siderophore receptors (2), receptors for iron-saturated transferrin (3), or receptors for iron-saturated lactoferrin (4), that allow them to remove and utilize iron from iron-binding proteins.

Whereas much has been learned about iron acquisition by extracellular pathogens, relatively little is known about iron acquisition by intracellular pathogens. We have been exploring some aspects of this subject using the intracellular bacterial pathogen *Legionella pneumophila*. This pathogen multiplies intracellularly in human mononuclear phagocytes; in the lung, it parasitizes alveolar macrophages and causes Legionnaires' disease. Cell-mediated immunity plays a major role in host defense against *L. pneumophila*, and activated monocytes and alveolar macrophages, including those activated by interferon gamma (IFN $\gamma$ )<sup>1</sup> inhibit *L. pneumophila* intracellular multiplication (5, 6). As multiplication of *L. pneumophila* is exclusively intracellular under tissue culture conditions (7), this organism is ideal for studies of iron acquisition by an intracellular pathogen, and of the role of iron in macrophage activation.

In previous studies, we have determined that *L. pneumophila* intracellular multiplication is iron dependent (8). We have also found that IFN $\gamma$ -activated monocytes inhibit *L. pneumophila* intracellular multiplication by limiting the availability of intracellular iron (8). Our studies have revealed two mechanisms by which IFN $\gamma$ -activated monocytes may accomplish this. First, they markedly downregulate transferrin receptors which bind and endocytize iron-saturated transferrin (8); the transferrin-transferrin receptor endocytic pathway is the

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1. Abbreviations used in this paper: FeNTA, ferric nitrilotriacetate; IFN $\gamma$ , recombinant human interferon gamma; NTA, sodium nitrilotriacetate; RPMI, RPMI 1640 medium with L-glutamine.

major physiologic conduit for iron into the cell. Second, IFN $\gamma$ -activated monocytes downregulate the concentration of intracellular ferritin (9), the major iron storage protein in the cell and a readily available source of mobilizable iron.

In the present study, we have explored the role of lactoferrin in *L. pneumophila*-monocyte interaction. Lactoferrin, like transferrin, is an iron binding protein in the human body which can increase the level of mononuclear phagocyte intracellular iron (10–12). Lactoferrin is felt to play an important role in host defense by acting to withhold iron from pathogenic bacteria during infection (13). By binding iron and then being internalized by mononuclear phagocytes, lactoferrin may contribute to the decrease in serum iron, the so-called hypoferremic response, seen in infection or inflammatory states (14). Although the effect of lactoferrin on the multiplication of extracellular pathogens has been well described (1), its effect on the multiplication of intracellular pathogens has not.

In contrast to iron-saturated transferrin, which enters a nonlysosomal intracellular compartment of the cell which has approximately the same relatively high pH as the *L. pneumophila* phagosome (~ 6.3) (15, 16), iron-saturated lactoferrin enters a lysosomal intracellular compartment of relatively low pH (< 5) (10–12). Because of these differences in the endocytic pathways of lactoferrin and transferrin, we wondered whether *L. pneumophila* would have access to iron derived from iron-lactoferrin as it does to iron derived from iron-transferrin.

In contrast to transferrin receptors, which have a low affinity for apotransferrin, lactoferrin receptors have a high affinity for apolactoferrin, which is evidently endocytized as efficiently as iron-saturated lactoferrin (17, 18). We therefore wondered whether apolactoferrin, a physiologic iron chelator, would act to limit the availability of iron to intracellular *L. pneumophila* in monocytes as does the nonphysiologic iron chelator deferoxamine.

Our studies shall show that *L. pneumophila* can utilize iron delivered into the infected monocyte via the lactoferrin-lactoferrin receptor endocytic pathway and that iron-saturated lactoferrin can reverse the capacity of IFN $\gamma$ -activated monocytes to inhibit *L. pneumophila* intracellular multiplication. In contrast, apolactoferrin inhibits *L. pneumophila* multiplication in nonactivated monocytes and enhances the capacity of IFN $\gamma$ -activated monocytes to inhibit *L. pneumophila* intracellular multiplication. These findings raise the possibility that at sites of inflammation, lactoferrin may inhibit or promote *L. pneumophila* intracellular multiplication in human monocytes depending upon its degree of iron saturation. Thus, apolactoferrin may limit bacterial multiplication in nonactivated and activated monocytes and boost host defense against *L. pneumophila* whereas iron-saturated lactoferrin may interfere with activation and be counterproductive to host defense against this pathogen.

In this study, we have also explored the role in *L. pneumophila*-monocyte interaction of nonphysiologic iron chelators that enter mononuclear phagocytes independently of the transferrin-lactoferrin receptor endocytic pathways. We wondered whether *L. pneumophila* would have access to iron that enters monocytes via a pathway that is not receptor mediated. Our studies shall demonstrate that *L. pneumophila* does have access to iron that enters the monocyte in this way and that three nonphysiologic iron compounds can reverse the capacity of IFN $\gamma$ -activated monocytes to inhibit *L. pneumophila* intracellular multiplication.

## Methods

**Media.** Egg yolk buffer with or without 1% BSA, and PBS were prepared or obtained as described (7). RPMI 1640 medium with L-glutamine (RPMI) (Gibco Laboratories, Grand Island, NY) was used in tissue culture experiments.

**Iron compounds.** In initial experiments, native human lactoferrin was used that was > 99% pure and 21% iron saturated according to the manufacturers specifications (Calbiochem-Behring Corp., La Jolla, CA). It was further iron saturated using ferric citrate, or desaturated using acetic acid/sodium acetate buffer with 40 mM EDTA and 0.2 M sodium phosphate at pH 4.0 as described (19). Saturated and unsaturated lactoferrin preparations were then dialyzed extensively against RPMI medium and filtered through 0.22- $\mu$ m filter units. In later experiments, iron-saturated and native lactoferrin were obtained from a different source (Sigma Chemical Co., St. Louis, MO). Preparations from the two sources were found to have identical biological effects in our assays. The iron-saturated preparations used in experiments were 90% saturated and the unsaturated preparations were 2–8% iron-saturated as determined by measuring absorbance at 465 nm using a value of 0.58 as the absorbance of a 1% solution of iron-saturated lactoferrin as the reference standard (19).

Ferric nitrilotriacetate (FeNTA) was prepared as previously described (20) and was prepared fresh for each experiment. Briefly, nitrilotriacetic acid (disodium salt; Sigma Chemical Co.) was dissolved in double distilled water and mixed in a 1:1 ratio with ferric chloride (Fisher Scientific Co., Fairlawn, NJ) that had been dissolved in 1 N HCL. The pH of the solution was then adjusted to neutrality with 1 N NaOH. Sodium nitrilotriacetate (NTA) was prepared in the same manner but without ferric chloride. Both preparations were filtered through a 0.22- $\mu$ m filter unit.

Ferric ammonium citrate (Sigma Chemical Co.) and ferric pyrophosphate (Sigma Chemical Co.) were dissolved in RPMI medium and then filtered through a 0.22- $\mu$ m filter unit.

**Agar.** Modified charcoal yeast extract agar was prepared in 100  $\times$  15-mm bacteriologic petri dishes as described (7).

**Bacteria.** *Legionella pneumophila*, Philadelphia 1 strain, serogroup 1, was grown in embryonated hens' eggs, harvested, tested for viability and for the presence of contaminating bacteria, and stored at  $-70^{\circ}\text{C}$ , as described (7). The bacteria were then passed one time only on modified charcoal yeast extract agar for 80 h, harvested, stored at  $-70^{\circ}\text{C}$ , and thawed in a  $37^{\circ}\text{C}$  waterbath just before use.

**Human blood mononuclear cells.** Mononuclear cells used in all experiments were obtained from the blood of healthy, adult volunteers with no history of Legionnaires' disease. The blood mononuclear cell fraction was obtained by centrifugation over a Ficoll-sodium diatrizoate solution (Pharmacia Fine Chemicals, Piscataway, NJ) as previously described (7).

**Serum.** Venous blood was obtained from healthy adult volunteers with no history of Legionnaires' disease. Serum was separated and used immediately. In all experiments, autologous serum was used.

**Assay for the effect of lactoferrin and iron compounds on *L. pneumophila* multiplication in nonactivated and IFN $\gamma$ -activated monocytes.** Freshly explanted monocytes were adhered to Linbro flat-bottomed wells (16 mm diameter; Flow Laboratories, Inc., McLean, VA) as described (21). The monocyte monolayers, containing  $\sim 5 \times 10^5$  cells, were then incubated in 0.5 ml RPMI containing 20% fresh normal human serum in 5% CO $_2$ -95% air at  $37^{\circ}\text{C}$  in the presence of human recombinant IFN $\gamma$  (2  $\mu\text{g/ml}$  equivalent to 20,000 U/ml) (Genentech, Inc., South San Francisco, CA) or control buffer (PBS-BSA). Nonactivated monocytes were incubated with iron-saturated lactoferrin (6 mg/ml) or apolactoferrin (2–3 mg/ml). IFN $\gamma$ -treated cultures were incubated with iron lactoferrin (0.07–6 mg/ml; 28%, 51%, or 90% iron saturated), apolactoferrin (2 mg/ml), FeNTA (3.4–112  $\mu\text{g/ml}$ ; 0.1–25  $\mu\text{g}$  iron/ml), NTA (87  $\mu\text{g/ml}$ ), ferric ammonium citrate (8 and 25  $\mu\text{g}$  iron/ml), ferric pyrophosphate (8 and 25  $\mu\text{g}$  iron/ml) or control buffer. After 24 h, *L. pneumophila* ( $10^4$  CFU/ml) were added to the monocyte cultures. The cultures were then incubated at  $37^{\circ}\text{C}$  in 5% CO $_2$ -95% air

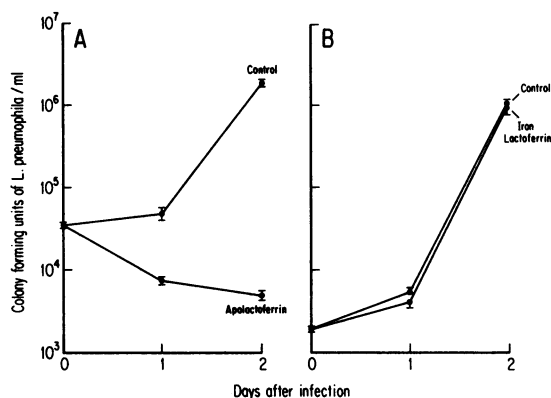
on a gyratory shaker (100 rpm) for 1 h and under stationary conditions thereafter. CFU of *L. pneumophila* in each culture were determined daily as described (7). At the end of each experiment, the viability of monocytes was assayed by trypan blue exclusion. All experiments were run in triplicate.

In some experiments, *L. pneumophila* were preincubated with or without apolactoferrin (3 mg/ml) in 0.5 ml RPMI containing 20% fresh normal human serum in 5% CO<sub>2</sub>-95% air at 37°C for 24 h. The bacteria were then washed thoroughly, resuspended in RPMI and added to monocyte cultures as in the experiments described above.

In experiments conducted under serumless conditions, freshly explanted monocytes were adhered to Linbro flat-bottomed wells in the presence of serum and then vigorously washed five times with Iscoves modified Dulbeccos medium (Gibco) to remove nonadherent cells and serum. The monocyte monolayers, containing ~ 5 × 10<sup>5</sup> cells, were then incubated in 0.5 ml of Iscoves medium at 37°C in 5% CO<sub>2</sub>-95% air in the presence of IFN $\gamma$  (2  $\mu$ g/ml) or control buffer (PBS-BSA). Monocytes treated with IFN $\gamma$  were also incubated with iron lactoferrin (3 mg/ml), ferric ammonium citrate (25  $\mu$ g iron/ml) or control media. After 24 h, the monocyte monolayers were infected with preopsonized *L. pneumophila* that had been incubated in 20% autologous normal human serum (NHS) for 30 min, washed twice by centrifugation at 15,000 g for 5 min in a refrigerated microcentrifuge (Savant Instruments, Inc., Hicksville, NY), and resuspended in Iscoves medium. CFU of *L. pneumophila* in each triplicate culture were determined as above.

## Results

*Apolactoferrin inhibits L. pneumophila intracellular multiplication in nonactivated monocytes.* *L. pneumophila* intracellular multiplication is iron dependent, and consequently, the iron-chelator deferoxamine inhibits *L. pneumophila* multiplication in human monocytes (8). To determine if apolactoferrin, a physiologic iron chelator recognized by lactoferrin receptors, also inhibits *L. pneumophila* multiplication in human monocytes, we infected monocytes in the presence or absence of this molecule. At a concentration of 2.0 mg/ml, apolactoferrin markedly inhibited *L. pneumophila* intracellular multiplication (Fig. 1 A). The inhibitory effect was not due to a toxic

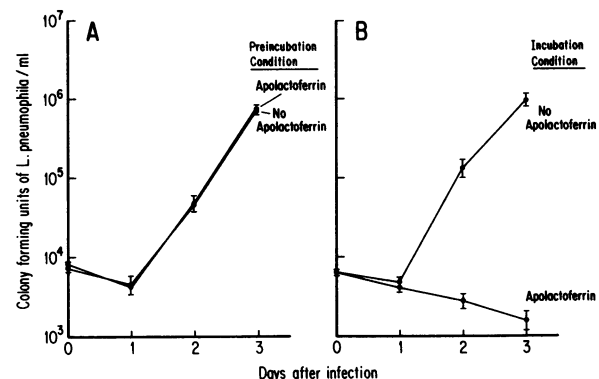


**Figure 1.** Apolactoferrin inhibits *L. pneumophila* intracellular multiplication in nonactivated human monocytes but iron-saturated lactoferrin has no effect. Monocytes in monolayer culture were incubated with apolactoferrin (2 mg/ml) or control medium (RPMI containing 20% NHS) (A) or iron-saturated lactoferrin (6 mg/ml) or control medium (B). After 24 h, all cultures were infected with *L. pneumophila*, and CFU were determined daily. Data are mean  $\pm$  SEM for triplicate cultures.

effect of apolactoferrin on monocytes because apolactoferrin-treated monocyte monolayers were intact after 4 d of culture and the monocytes were viable by trypan blue exclusion (data not shown).

The inhibitory effect of apolactoferrin was dependent upon its iron-unsaturated state. In contrast to apolactoferrin, iron-saturated lactoferrin, at a concentration of 6 mg/ml, had no effect on *L. pneumophila* multiplication (Fig. 1 B).

*The inhibitory effect of apolactoferrin on L. pneumophila intracellular multiplication is due to its interaction with the monocyte and not to a direct toxic effect on the bacterium.* Apolactoferrin has been found to kill gram-negative bacteria under hypoosmolar conditions (22, 23). In previous studies by other investigators, apolactoferrin has been found to exert a bactericidal effect against broth-grown (but not agar-grown) *L. pneumophila* suspended in water (24, 25). Although the experimental conditions of these studies were very different from ours, e.g., we used agar-grown bacteria and held them under physiologic conditions (isosmolar medium buffered to pH 7.4, 37°C, 5% CO<sub>2</sub>-95% air), we performed additional experiments to explore the possibility that apolactoferrin was directly toxic to *L. pneumophila* under our experimental conditions. First, we incubated *L. pneumophila* in tissue culture medium with and without apolactoferrin (3 mg/ml) for 3 d and assessed bacterial survival. Apolactoferrin had no influence on the survival of *L. pneumophila* in tissue culture medium (data not shown). Second, we examined the possibility that apolactoferrin exerted an effect on the bacteria which subsequently rendered them more susceptible to killing or less able to multiply in the monocyte. We preincubated the bacteria with or without apolactoferrin for 24 h, washed them, added them to monocytes, and assessed their capacity to multiply. Preincubation of *L. pneumophila* with apolactoferrin had no influence on the capacity of the bacteria to multiply in monocytes (Fig. 2 A). In contrast, when *L. pneumophila* from the same stock were added to replicate monocyte monolayers in the same experiment, but in the pres-



**Figure 2.** Apolactoferrin is not directly toxic to *L. pneumophila* but exerts its inhibitory effect on *L. pneumophila* intracellular multiplication via an interaction with the monocyte. *L. pneumophila* were incubated with or without apolactoferrin (3 mg/ml) in tissue culture medium (RPMI containing 20% NHS) for 24 h, washed, and incubated with monocytes in monolayer culture in the absence of apolactoferrin (A). In the same experiment, untreated *L. pneumophila* from the same stock used in A were incubated with replicate monocyte cultures in the presence or absence of apolactoferrin (3 mg/ml) (B). CFU were determined daily. Data are mean  $\pm$  SEM for triplicate cultures.

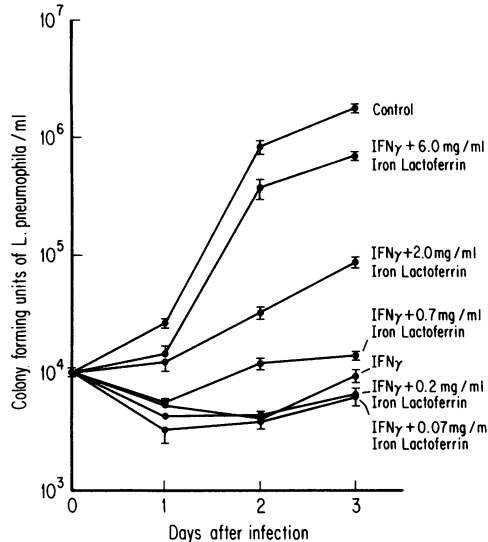
ence of apolactoferrin, *L. pneumophila* intracellular multiplication was inhibited (Fig. 2 B).

These results indicated that apolactoferrin is not directly toxic to *L. pneumophila* and that it exerts its inhibitory effect on *L. pneumophila* intracellular growth via an interaction with the monocyte. Thus, apolactoferrin appears to limit the availability of iron to intracellular *L. pneumophila* and thereby restrict bacterial multiplication in a manner similar to deferoxamine in previous studies (8).

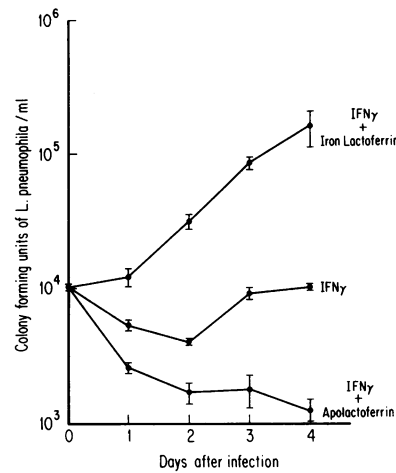
*Iron-saturated lactoferrin reverses, whereas apolactoferrin enhances, the capacity of activated monocytes to inhibit L. pneumophila intracellular multiplication.* IFN $\gamma$ -activated monocytes inhibit *L. pneumophila* intracellular multiplication by limiting the availability of iron to the bacterium (8). To determine if iron lactoferrin could supply iron to *L. pneumophila* and reverse the inhibitory effect of activation, we activated monocytes with IFN $\gamma$  in the presence of various concentrations of iron lactoferrin, infected them with *L. pneumophila*, and assayed intracellular multiplication (Fig. 3).

Iron-lactoferrin at a concentration of 6 mg/ml reversed the capacity of IFN $\gamma$ -activated monocytes to inhibit *L. pneumophila* multiplication. This neutralizing effect of iron lactoferrin on monocyte activation was dose dependent (Fig. 3). At a concentration of 2.0 and 0.7 mg/ml, iron lactoferrin partially reversed monocyte activation. At concentrations of 0.2 mg/ml or less, iron lactoferrin had little or no effect on the capacity of activated monocytes to inhibit *L. pneumophila* multiplication.

To confirm that the effect of iron lactoferrin was due to iron and not lactoferrin, we compared the effect of iron lactoferrin to that of apolactoferrin on the capacity of IFN $\gamma$ -activated monocytes to inhibit *L. pneumophila* multiplication (Fig. 4).



**Figure 3.** Iron-saturated lactoferrin reverses the capacity of IFN $\gamma$ -activated human monocytes to inhibit *L. pneumophila* intracellular multiplication in a dose-dependent fashion. Monocytes in monolayer culture were incubated in medium alone (RPMI containing 20% NHS) (control) or in medium containing IFN $\gamma$  to activate them. IFN $\gamma$ -treated monocytes were additionally incubated with various concentrations of iron-saturated lactoferrin (0–6.0 mg/ml), as indicated. After 24 h, all cultures were infected with *L. pneumophila* and CFU were determined daily. Data are mean $\pm$ SEM for triplicate cultures.



**Figure 4.** Iron-saturated lactoferrin reverses and apolactoferrin enhances the capacity of IFN $\gamma$ -activated monocytes to inhibit *L. pneumophila* intracellular multiplication. Monocytes were activated with IFN $\gamma$  and, at the same time, they were incubated with control medium (RPMI containing 20% NHS), iron-saturated lactoferrin (2 mg/ml), or apolactoferrin (2 mg/ml). After 24 h, all cultures were infected with *L. pneumophila*, and CFU were determined daily. Data are mean $\pm$ SEM for triplicate cultures.

Figure 4. Iron-saturated lactoferrin reverses and apolactoferrin enhances the capacity of IFN $\gamma$ -activated monocytes to inhibit *L. pneumophila* intracellular multiplication. Monocytes were activated with IFN $\gamma$  and, at the same time, they were incubated with control medium (RPMI containing 20% NHS), iron-saturated lactoferrin (2 mg/ml), or apolactoferrin (2 mg/ml). After 24 h, all cultures were infected with

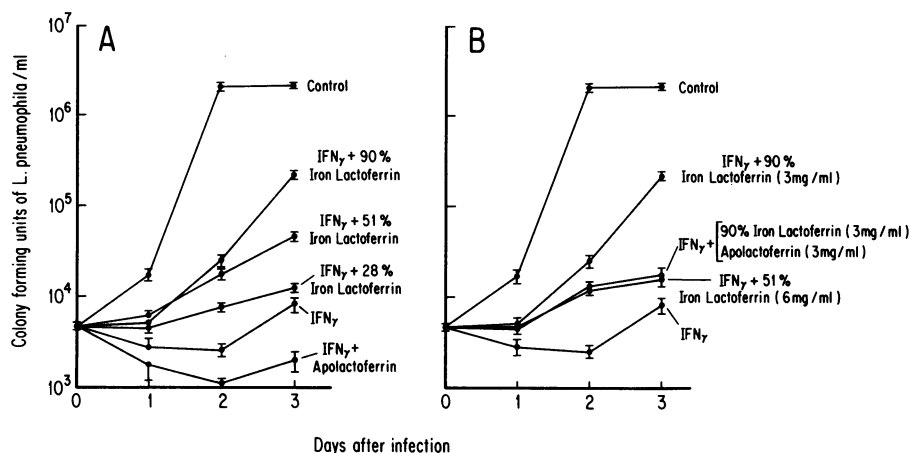
*L. pneumophila*, and CFU were determined daily. Data are mean $\pm$ SEM for triplicate cultures.

At concentrations of apolactoferrin > 2.0 mg/ml, the activated monocytes did not remain adherent to the tissue culture wells, so we studied the effects of iron-lactoferrin and apolactoferrin at a concentration of 2.0 mg/ml. Whereas iron-lactoferrin partially reversed the inhibitory effect of activation, apolactoferrin had no such effect and in fact enhanced the inhibitory effect of IFN $\gamma$ . In these experiments, the apolactoferrin-treated monolayers remained intact throughout the course of the infection, and the monocytes were viable at 4 d by trypan blue exclusion.

These experiments show that iron-lactoferrin is capable of reversing the inhibitory effect of IFN $\gamma$  activation on *L. pneumophila* intracellular multiplication and that this effect is due to iron and not the lactoferrin molecule. These experiments also show that apolactoferrin enhances the capacity of IFN $\gamma$ -activated monocytes to inhibit *L. pneumophila* intracellular multiplication.

*The capacity of iron-lactoferrin to reverse the inhibitory effect of activated monocytes on L. pneumophila intracellular multiplication is dependent upon its degree of iron saturation and not only its total iron content.* To determine the influence of iron-saturation on the capacity of iron-lactoferrin to reverse the inhibitory effect of activated monocytes, we activated monocytes with IFN $\gamma$  in the presence of 3 mg/ml lactoferrin that was 90, 51, or 28% iron saturated, infected them with *L. pneumophila*, and assayed intracellular multiplication. The capacity of iron-lactoferrin to reverse the inhibitory effect of monocyte activation was dependent upon its percent saturation (90 > 51 > 28%) (Fig. 5 A).

The greater capacity of more saturated iron-lactoferrin to reverse monocyte activation against *L. pneumophila* might simply reflect its higher iron content. In addition, the ratio of saturated to unsaturated lactoferrin might be an important factor. To examine this possibility, we activated monocytes with IFN $\gamma$  in the presence of three different iron lactoferrin preparations containing nearly comparable amounts of total iron but two very different ratios of saturated:unsaturated lactoferrin (Fig. 5 B). The preparation containing a high ratio (9:1) of saturated:unsaturated lactoferrin (3 mg/ml of 90% iron lactoferrin) had a significantly greater capacity to reverse monocyte activation than two preparations containing a low ratio (~ 1:1) of saturated:unsaturated lactoferrin (3 mg/ml of 90% iron lac-



**Figure 5.** The capacity of iron-lactoferrin to reverse monocyte activation against *L. pneumophila* is dependent on its degree of iron saturation and not only its iron content. Monocytes were incubated in medium alone (RPMI containing 20% NHS) (control) or activated with IFN $\gamma$ . In *A*, IFN $\gamma$ -treated monocytes were also incubated with a fixed amount of lactoferrin (3 mg/ml) that was saturated with various concentrations of iron (90, 51, or 28%) or with the same amount of apolactoferrin. In *B*, IFN $\gamma$ -treated monocytes were also incubated with a fixed amount of iron but two different ratios of saturated:unsaturated lactoferrin, 9:1 (3 mg/ml of 90% iron-lactoferrin) or 1:1 (3 mg/ml of 90% iron-lactoferrin plus 3 mg/ml apolactoferrin); 6

mg/ml of 51% iron-lactoferrin). In *A* and *B*, one set of IFN $\gamma$ -treated monocytes were incubated without lactoferrin. After 24 h, all cultures were infected with *L. pneumophila*, and CFU were determined daily. Data are mean $\pm$ SEM for triplicate cultures.

toferrin plus 3 mg/ml of apolactoferrin; 6 mg/ml of 51% iron-lactoferrin). Viewed another way, apolactoferrin neutralized the capacity of highly saturated (90%) iron-lactoferrin to reverse monocyte activation against *L. pneumophila*. Thus, the percent iron saturation of lactoferrin and not just its total iron content is an important determinant of its capacity to reverse monocyte activation against *L. pneumophila*.

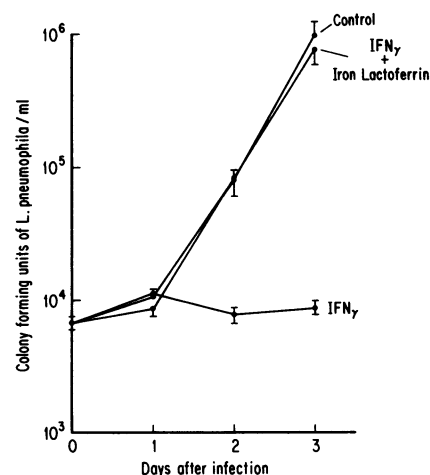
*Iron-lactoferrin reverses the inhibitory effect of monocyte activation by a transferrin receptor-independent route.* In the assays described above, monocytes were cultured in the presence of 20% normal human serum which contains transferrin. Although lactoferrin has a 300-fold higher affinity for iron than transferrin (26) the possibility remained that some exchange of iron from lactoferrin to transferrin occurred under these tissue culture conditions and that the effect of iron lactoferrin was mediated by iron-transferrin. To explore this possibility, we studied the effect of lactoferrin on monocyte activation in the absence of serum, and therefore of transferrin. We activated monocyte monolayers with IFN $\gamma$  in serumless medium, infected them with preopsonized *L. pneumophila* in the complete absence of serum, and assayed *L. pneumophila* intracellular multiplication (Fig. 6). In serumless medium, as in medium containing serum, IFN $\gamma$ -activated monocytes inhibited *L. pneumophila* intracellular multiplication. Iron-saturated lactoferrin (6 mg/ml) completely reversed this inhibitory effect. This experiment shows that iron-saturated lactoferrin can enter the monocyte via a transferrin receptor-independent route and supply iron to intracellular *L. pneumophila*.

*The nonphysiologic iron chelates FeNTA and ferric ammonium citrate reverse the capacity of activated monocytes to inhibit L. pneumophila intracellular multiplication.* We next explored whether nonphysiologic iron chelates that enter monocytes independently of the transferrin-transferrin receptor or lactoferrin-lactoferrin receptor pathways can reverse the capacity of IFN $\gamma$ -activated monocytes to inhibit *L. pneumophila* multiplication. We infected monocytes with *L. pneumophila* in the presence or absence of the nonphysiologic iron chelates FeNTA, ferric ammonium citrate, or ferric pyrophosphate and assayed intracellular multiplication (Fig. 7).

At an iron concentration of 8  $\mu$ g/ml, all three iron chelates partially reversed the inhibitory effect of IFN $\gamma$ -activated mono-

cytes on *L. pneumophila* multiplication (Fig. 7 *A*). At an iron concentration of 25  $\mu$ g/ml, FeNTA and ferric ammonium citrate completely reversed the inhibitory effect of IFN $\gamma$ -activated monocytes, but ferric pyrophosphate still only partially reversed the inhibitory effect (Fig. 7 *B*). The differences among these iron chelates in reversing the effect of monocyte activation probably reflect differences in their solubility at neutral pH and consequently their uptake by monocytes.

As was the case with iron lactoferrin, the neutralizing effect of FeNTA on monocyte activation was dose dependent (Fig. 8). At an iron concentration of 25  $\mu$ g/ml, FeNTA completely reversed the capacity of IFN $\gamma$ -activated monocytes to inhibit *L. pneumophila* multiplication. At iron concentrations of 8, 3, and 1  $\mu$ g/ml, FeNTA partially reversed monocyte activation.



**Figure 6.** Iron-saturated lactoferrin reverses the capacity of IFN $\gamma$ -activated human monocytes to inhibit *L. pneumophila* multiplication under serumless conditions. Monocytes were incubated in serumless medium alone (control) or they were activated by incubation in serumless medium containing IFN $\gamma$ . The IFN $\gamma$ -treated monocytes were additionally incubated with control medium or iron-lactoferrin (6 mg/ml). After 24 h, all cultures were infected with *L. pneumophila*, and CFU were determined daily. Data are mean $\pm$ SEM for triplicate cultures.

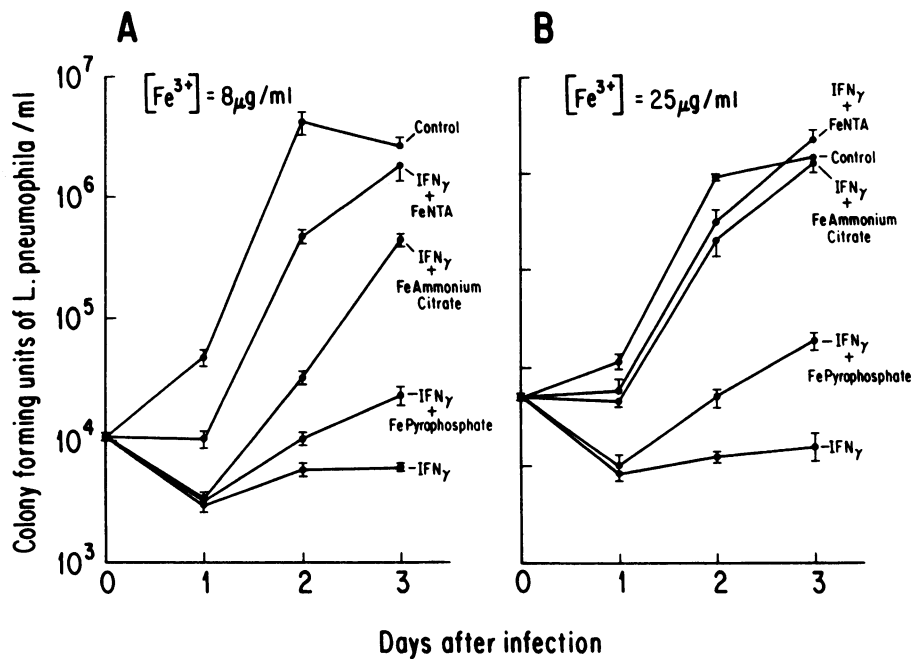


Figure 7. Nonphysiologic iron chelates reverse the capacity of IFN<sub>γ</sub>-activated monocytes to inhibit *L. pneumophila* intracellular multiplication. Monocytes were incubated in medium alone (RPMI containing 20% NHS) (control), or they were activated by incubation with IFN<sub>γ</sub>. At the same time, the IFN<sub>γ</sub>-treated monocytes were incubated with FeNTA, ferric ammonium citrate, or ferric pyrophosphate at iron concentrations of 8 µg/ml (A) or 25 µg/ml (B). After 24 h, all cultures were infected with *L. pneumophila*, and CFU were determined daily. Data are mean±SEM for triplicate cultures.

At iron concentrations of 0.3 µg/ml or less, ferric nitrilotriacetate had little or no effect on the capacity of activated monocytes to inhibit *L. pneumophila* multiplication.

To confirm that the effect of FeNTA was due to iron and not to nitrilotriacetate, we compared the effect of FeNTA to that of NTA on the capacity of IFN<sub>γ</sub>-activated monocytes to inhibit *L. pneumophila* multiplication (Fig. 9). Whereas FeNTA completely reversed the inhibitory effect of activation, NTA had no effect. Analysis by trypan blue exclusion showed that NTA-treated monolayers were intact and viable at 4 d.

These experiments demonstrated that the nonphysiologic iron chelates FeNTA and ferric ammonium citrate are capable of reversing the inhibitory effect of IFN<sub>γ</sub> on *L. pneumophila*

intracellular multiplication, and that at least in the case of FeNTA this effect is due to iron and not nitrilotriacetate.

The nonphysiologic iron chelates enter the monocyte and reverse the inhibitory effect of monocyte activation by a transferrin receptor-independent route. In the assays described above, monocytes were cultured in the presence of 20% normal human serum, which contains transferrin. The amount of iron-saturated transferrin in 20% serum, ~ 0.2 mg/ml, is below the level that even partially reverses monocyte activation against *L. pneumophila*. However, both FeNTA and ferric ammonium citrate can donate iron to transferrin, raising the possibility that the effect of these iron chelators may at least in part be mediated by the transferrin-transferrin receptor endocytic pathway. To explore this possibility, we studied the effect of the nonphysiologic iron chelates on monocyte activation in the absence of serum. We cultured monocytes in serumless media,

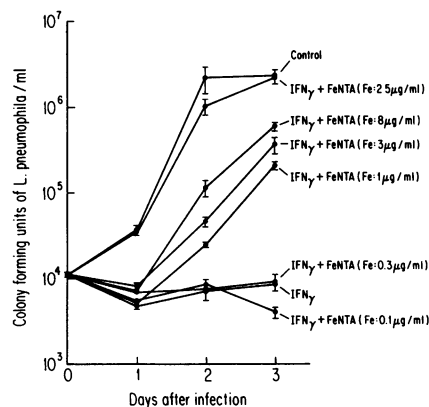


Figure 8. FeNTA reverses the capacity of IFN<sub>γ</sub>-activated monocytes to inhibit *L. pneumophila* intracellular multiplication in a dose-dependent fashion. Monocytes were incubated in medium alone (RPMI containing 20% NHS) (control), or they were activated by incubation with IFN<sub>γ</sub>. At the same time, the IFN<sub>γ</sub>-treated monocytes were incubated with various concentrations of FeNTA (0–25 µg iron/ml). After 24 h, all cultures were infected with *L. pneumophila*, and CFU were determined daily. Data are mean±SEM for triplicate cultures.

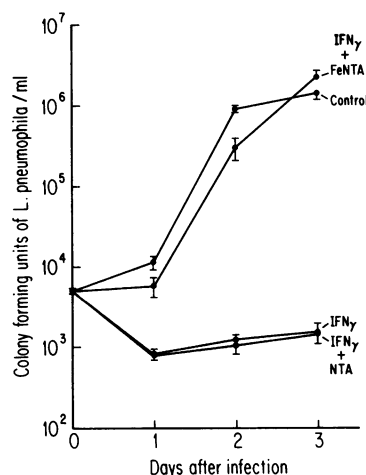


Figure 9. FeNTA, but not NTA reverses the capacity of IFN<sub>γ</sub>-activated human monocytes to inhibit *L. pneumophila* intracellular multiplication. Monocytes were incubated in medium alone (RPMI containing 20% NHS) (control), or they were activated by incubation with IFN<sub>γ</sub>. At the same time, the IFN<sub>γ</sub>-treated monocytes were incubated with FeNTA (25 µg iron/ml) or with an equivalent concentration of NTA without iron. After 24 h, all cultures were infected with *L. pneumophila*

and CFU were determined daily. Data are mean±SEM for triplicate cultures.

infected them with preopsonized *L. pneumophila* in the complete absence of serum, and assayed *L. pneumophila* intracellular multiplication (Fig. 10).

In the absence of serum, as in its presence, ferric ammonium citrate, at a concentration of 25  $\mu\text{g}$  iron/ml, completely reversed the inhibitory effect of IFN $\gamma$ -activated monocytes on *L. pneumophila* intracellular multiplication. Experiments using FeNTA could not be carried out under the serumless conditions employed, because this chelate was unstable and formed iron precipitates in the serumless medium.

These experiments show that the nonphysiologic iron chelate ferric ammonium citrate can enter the monocyte via a transferrin receptor-independent route and supply iron to intracellular *L. pneumophila*.

## Discussion

This study demonstrates that unsaturated lactoferrin, an iron-binding protein postulated to play a role in host defense against extracellular pathogens (1, 13, 22, 27), can inhibit *L. pneumophila* intracellular multiplication in nonactivated monocytes. In addition, this study shows that lactoferrin can reverse or enhance the capacity of IFN $\gamma$ -activated human monocytes to inhibit *L. pneumophila* intracellular multiplication depending on its degree of iron saturation.

Lactoferrin is an iron-binding protein with a high degree of homology to transferrin (26). It is found in the specific granules of neutrophils (28, 29) and in mucosal secretions (30) including bronchial secretions (31–33). In its unsaturated form, it has been found to have either a bacteriostatic or a bactericidal effect against a variety of extracellular bacteria in vitro (1, 13, 22, 23, 27). At sites of inflammation, such as occurs at infected sites in the body, unsaturated lactoferrin is released by the specific granules of neutrophils into the extracellular environment. At the acidic pH often found at sites of inflammation, free iron is present because transferrin is unable to bind and hold iron under acidic conditions. As lactoferrin has a high affinity for iron under acidic conditions it can bind this free iron, rendering it unavailable to pathogenic microorganisms

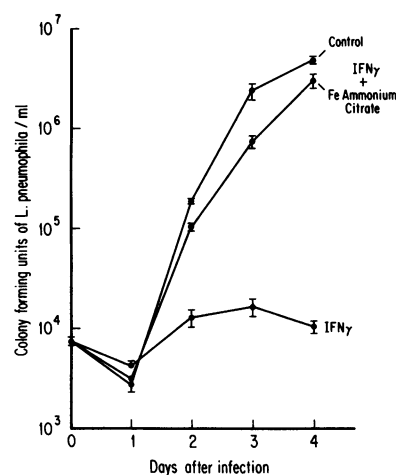


Figure 10. The nonphysiologic iron chelate ferric ammonium citrate reverses the capacity of IFN $\gamma$ -activated human monocytes to inhibit *L. pneumophila* multiplication under serumless conditions. Monocytes were incubated in serumless medium alone (control) or they were activated by incubation in serumless medium containing IFN $\gamma$ . The IFN $\gamma$ -treated monocytes were additionally incubated with control medium or

ferric ammonium citrate (25  $\mu\text{g}$  iron/ml). After 24 h, all cultures were infected with *L. pneumophila*, and CFU were determined daily. Data are mean  $\pm$  SEM for triplicate cultures.

(34). Iron-lactoferrin is taken up by mononuclear phagocytes via receptors with specificity for lactoferrin (17, 18). By this mechanism, lactoferrin contributes to a localized hypoferrremia at sites of infection or inflammation, and it may also contribute to the systemic hypoferrremia seen in these states (14).

The release of iron from lactoferrin within the mononuclear phagocyte appears to involve a different mechanism than the release of iron from transferrin. In the case of transferrin, endocytized iron transferrin enters a distinct, mildly acidic endosomal compartment within the cell where it releases iron and is then recycled back to the cell surface (15, 16). Whereas iron-lactoferrin also cycles through a mildly acidic endosomal compartment, it does not release iron under this condition. Iron is released from lactoferrin when a portion of the endocytized lactoferrin enters the lysosomal compartment and is degraded (10).

Although the incorporation of iron into the monocyte from iron-lactoferrin would appear to be less efficient than that from iron-transferrin, the kinetics of *L. pneumophila* multiplication in the presence of iron-transferrin (8) or iron-lactoferrin are similar given equivalent amounts of iron. Possibly, the much higher number of lactoferrin than transferrin receptors on the monocyte compensates for the difference between iron-lactoferrin and iron-transferrin in the efficiency of iron incorporation into the monocyte, and the two iron proteins thus have a similar net effect on *L. pneumophila* intracellular multiplication.

Although transferrin and lactoferrin are endocytized by different receptors and are transported through different intracellular pathways, the iron released from each of these compounds converges in the cell in the intermediate labile iron pool. Such iron is in a readily transportable form and is immediately available to the cell for metabolic processes or is shuttled to the intracellular iron storage protein ferritin. The intracellular shuttle system which transports iron in this pool is as yet uncharacterized (35). The evidence that iron from both lactoferrin and transferrin enters this pool is that iron entering through both these pathways is incorporated into intracellular ferritin (10–12, 35, 36). Our finding that intracellular *L. pneumophila* can utilize iron derived from iron-lactoferrin indicates that *L. pneumophila* has access to iron released by iron-lactoferrin to this pool.

Apolactoferrin inhibits the intracellular multiplication of *L. pneumophila* in nonactivated monocytes and enhances the inhibitory effect of activation on *L. pneumophila* intracellular multiplication. This enhancement is not seen when apotransferrin is added to activated monocytes (8). This may reflect more efficient endocytosis of lactoferrin because the affinity of the lactoferrin receptor for apolactoferrin is much greater than the affinity of the transferrin receptor for apotransferrin (17, 18, 37). In addition, whereas IFN $\gamma$  activation downregulates the expression of monocyte transferrin receptors (8), recent data suggests that IFN $\gamma$  has no effect on lactoferrin receptor expression (17). Moreover, lactoferrin may bind iron more tightly than transferrin under acidic conditions within the cell. Apolactoferrin may consequently exert an inhibitory effect on *L. pneumophila* growth by chelating iron in the labile iron pool of the host cell and thereby deprive the bacteria of iron. Similarly, we have found that the iron chelator deferoxamine inhibits *L. pneumophila* intracellular multiplication in human monocytes in vitro (8).



In our study, iron lactoferrin concentrations of 0.7 and 2.0 mg/ml partially, and 6 mg/ml completely reversed monocyte activation against *L. pneumophila*, and apolactoferrin concentrations of 2.0 mg/ml inhibited *L. pneumophila* intracellular multiplication. Whereas the local concentrations of lactoferrin at sites of inflammation are not known, milligrams-per-milliliter concentrations seem likely given that concentrations of lactoferrin in human plasma during infection can exceed 0.2 mg/ml (38). PMN are prominent in acute bacterial infection of the lung, and in an experimental murine model of pneumonia, increased concentrations of lactoferrin were demonstrated in lung lavage fluid, primarily as a result of neutrophil degranulation (39, 40). PMN are also prominent in *L. pneumophila* infection of the lung, which is characterized histologically by an initial influx of PMN followed by an influx of mononuclear phagocytes, such that most established cases of pneumonia demonstrate a mix of neutrophils and mononuclear phagocytes (41). As already noted, lactoferrin is not only released by neutrophils (14), it is also synthesized by bronchial glands in the respiratory tract (30–33) and is present in bronchoalveolar lavage fluid (42, 43). Epithelial cell-derived lactoferrin appears to be responsible for much of the lactoferrin present in the lower respiratory tract of normal human subjects and patients with chronic bronchitis (44). In patients with pulmonary diseases such as cystic fibrosis and bronchitis, lactoferrin is present in pooled pulmonary secretions in concentrations as high as 1.0–2.5 mg/ml (32, 33). Thus, it seems conceivable that in a *L. pneumophila* pneumonic infiltrate, lactoferrin from neutrophils and bronchial secretions attains concentrations sufficient to influence monocyte activation in our study.

The capacity of lactoferrin to mediate growth of *L. pneumophila* in mononuclear phagocytes is dependent upon not only its iron content but also its percent iron saturation. Thus, in our study, highly iron-saturated lactoferrin strongly reversed monocyte activation against *L. pneumophila*, whereas poorly iron-saturated lactoferrin in an amount that had an equivalent total content of iron did not. These results imply that the net delivery of iron by lactoferrin into the mononuclear phagocyte is the result of an equilibrium between iron lactoferrin molecules entering the cell and releasing iron, and apolactoferrin molecules entering the cell, binding iron, and removing it from the cell.

Our data suggest that lactoferrin, when released at sites of inflammation in the *L. pneumophila*-infected lung, may augment or impede host defense against *L. pneumophila* depending upon its degree of iron saturation. Information on the percent saturation of lactoferrin in the *L. pneumophila*-infected lung is not available. When initially released by neutrophils, lactoferrin has been reported to be 8% saturated with iron (14). The percent iron saturation of lactoferrin released by other cell types at mucosal surfaces is not known. Nor is it known how rapidly apolactoferrin acquires iron at sites of inflammation. Consequently, whether lactoferrin acts in a way that enhances or is counterproductive to host defense against *L. pneumophila* is not clear.

Our study demonstrates that, in addition to iron-transferrin and iron-lactoferrin, nonphysiologic iron chelates including FeNTA can supply iron to *L. pneumophila* and neutralize the capacity of activated monocytes to inhibit *L. pneumophila* intracellular multiplication. Interestingly, FeNTA does not necessarily substitute for iron-transferrin or iron-lactoferrin in other

situations. Whereas iron-transferrin, iron-lactoferrin, and FeNTA all increase the intracellular level of lymphocyte iron, only iron transferrin or iron lactoferrin provides iron in a form that allows proliferation of stimulated lymphocytes to occur (45, 46). Similarly, iron transferrin but not FeNTA stimulates the proliferation and differentiation of renal tubular cells (47). This may reflect different distributions and activities of physiologic and nonphysiologic iron compounds in cells. Along this line, Chang cells incorporate iron when treated with iron-transferrin or FeNTA but distribute the iron differently intracellularly (48).

In our study, all three nonphysiologic iron compounds studied at least partially neutralized monocyte activation against *L. pneumophila*. FeNTA was the most effective compound in that it almost completely reversed activation at 8  $\mu$ g iron/ml. Ferric ammonium citrate was the next most effective compound; it was only partially effective at 8  $\mu$ g iron/ml but it completely reversed activation at 25  $\mu$ g iron/ml. Ferric pyrophosphate was the least effective, only partially reversing activation even at 25  $\mu$ g iron/ml. The differences among these compounds may reflect differences in their solubility properties. FeNTA, the most effective compound, has unique solubility properties among iron chelates. While most iron chelates polymerize at neutral or alkaline pH, FeNTA remains fully soluble in this pH range (20). Consequently, it remains highly soluble at the near neutral pH (7.4) of tissue culture media.

Our study shows that monocytes cultured under serumless conditions are able to support *L. pneumophila* intracellular multiplication, and that treatment of these cells with IFN $\gamma$  activates them such that they inhibit *L. pneumophila* intracellular multiplication. This indicates that monocytes possess enough endogenous iron to support *L. pneumophila* multiplication in the absence of exogenous iron. The capacity of ferric ammonium citrate to reverse monocyte activation under serumless conditions indicates that activated monocytes limit iron availability to intracellular *L. pneumophila* by mechanisms additional to downregulation of transferrin receptors. Consistent with this view, we have recently found that IFN $\gamma$ -activated monocytes markedly decrease the concentration of the iron storage protein ferritin (9). The capacity of ferric ammonium citrate to reverse monocyte activation under serumless conditions also confirms that *L. pneumophila* utilize iron that enters monocytes independently of the transferrin-transferrin receptor or lactoferrin-lactoferrin receptor endocytic pathways.

The mechanism by which nonphysiologic iron chelates enter the cell in the absence of physiologic iron-binding proteins is unclear. One mechanism might simply be fluid phase endocytosis. Another mechanism is suggested by studies of iron transport into hepatocytes indicating that nontransferrin-bound iron is transported into these cells by a membrane carrier for iron, and that this process is driven by the membrane potential difference between the interior and exterior of the cell (49).

Our study confirms the central role of iron in IFN $\gamma$ -mediated monocyte activation against *L. pneumophila*. Our finding that a variety of iron compounds, both physiologic and nonphysiologic, can reverse monocyte activation against *L. pneumophila*, and that a nonphysiologic iron chelate can reverse activation even under serumless conditions, emphasizes that iron, and not its carrier molecule, is responsible for the



neutralizing effect of these iron compounds on monocyte activation.

Our study suggests a potential role for PMN, which are prominent at sites of infection in the lungs of patients with Legionnaires' disease, in host defense against *L. pneumophila*. Previous studies from this laboratory have indicated that PMN lack the capacity to kill appreciable numbers of *L. pneumophila* even in the presence of specific antibody and complement (50). However, the current study provides a potential indirect role for PMN in host defense against *L. pneumophila*: providing infected mononuclear phagocytes with apolactoferrin and thereby enabling them to inhibit *L. pneumophila* intracellular multiplication. Consistent with this idea, a previous study has demonstrated that peritoneal macrophages from mice infected intraperitoneally with various mycobacteria contain lactoferrin, evidently derived from PMN, and that the addition of granulocytic extract to peritoneal macrophages infected with mycobacteria enhances their capacity to restrict mycobacterial growth in vitro (51).

In a broader sense, our study supports the concept that PMN and mononuclear phagocytes may cooperate in host defense against intracellular parasites and other pathogens. PMN or their granule products have been reported to enhance the antimicrobial capacity of mononuclear phagocytes against a variety of pathogens in addition to *L. pneumophila* and mycobacteria. These include *Toxoplasma gondii* (52), *Trypanosoma cruzi* (53), *Staphylococcus aureus* (54), and *Candida albicans* (55). In addition to apolactoferrin, eosinophil peroxidase has been implicated as playing a role in PMN-mononuclear phagocyte cooperation; mononuclear phagocytes have been shown to have an enhanced capacity to kill eosinophil peroxidase-coated *T. gondii*, *T. cruzi*, and *S. aureus* (52-54).

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