

Chloroquine inhibits the intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron. A potential new mechanism for the therapeutic effect of chloroquine against intracellular pathogens.

T F Byrd, M A Horwitz

J Clin Invest. 1991;**88**(1):351-357. <https://doi.org/10.1172/JCI115301>.

Research Article

Chloroquine and ammonium chloride, by virtue of their basic properties, have been shown to raise endocytic and lysosomal pH and thereby interfere with normal iron metabolism in a variety of cell types, including mononuclear phagocytes. Cellular iron metabolism is of critical importance to *Legionella pneumophila*, an intracellular bacterial pathogen whose capacity to multiply in human mononuclear phagocytes is dependent upon the availability of intracellular iron. In view of this, we have studied the effects of chloroquine and ammonium chloride on *L. pneumophila* intracellular multiplication in human monocytes. Chloroquine, at a concentration of 20 microM, and ammonium chloride, at a concentration of 20 mM, inhibited *L. pneumophila* intracellular multiplication by 1.4 +/- 0.2 (SEM) logs and 1.5 +/- 0.2 logs, respectively. Chloroquine- and ammonium chloride-induced inhibition of *L. pneumophila* intracellular multiplication was completely reversed by iron nitrilotriacetate, an iron compound which is soluble in the neutral to alkaline pH range, but not by iron transferrin, which depends upon acidic intracellular conditions to release iron. Chloroquine had no major direct effect on *L. pneumophila* multiplication in artificial media except at extremely high concentrations (15,000-fold that which inhibited *L. pneumophila* multiplication in mononuclear phagocytes), and inhibition at such concentrations was not reversed by iron nitrilotriacetate. This study demonstrates that chloroquine and ammonium chloride inhibit the intracellular multiplication of *L. pneumophila* by limiting the [...]

Find the latest version:

<https://jci.me/115301/pdf>



Chloroquine Inhibits the Intracellular Multiplication of *Legionella pneumophila* by Limiting the Availability of Iron

A Potential New Mechanism for the Therapeutic Effect of Chloroquine against Intracellular Pathogens

Thomas F. Byrd and Marcus A. Horwitz

Division of Infectious Diseases, Department of Medicine, School of Medicine, University of California Los Angeles, Los Angeles, California 90026

Abstract

Chloroquine and ammonium chloride, by virtue of their basic properties, have been shown to raise endocytic and lysosomal pH and thereby interfere with normal iron metabolism in a variety of cell types, including mononuclear phagocytes. Cellular iron metabolism is of critical importance to *Legionella pneumophila*, an intracellular bacterial pathogen whose capacity to multiply in human mononuclear phagocytes is dependent upon the availability of intracellular iron. In view of this, we have studied the effects of chloroquine and ammonium chloride on *L. pneumophila* intracellular multiplication in human monocytes.

Chloroquine, at a concentration of 20 μ M, and ammonium chloride, at a concentration of 20 mM, inhibited *L. pneumophila* intracellular multiplication by 1.4 ± 0.2 (SEM) logs and 1.5 ± 0.2 logs, respectively. Chloroquine- and ammonium chloride-induced inhibition of *L. pneumophila* intracellular multiplication was completely reversed by iron nitrilotriacetate, an iron compound which is soluble in the neutral to alkaline pH range, but not by iron transferrin, which depends upon acidic intracellular conditions to release iron. Chloroquine had no major direct effect on *L. pneumophila* multiplication in artificial media except at extremely high concentrations (15,000-fold that which inhibited *L. pneumophila* multiplication in mononuclear phagocytes), and inhibition at such concentrations was not reversed by iron nitrilotriacetate.

This study demonstrates that chloroquine and ammonium chloride inhibit the intracellular multiplication of *L. pneumophila* by limiting the availability of iron to the bacterium. It is possible that such a mechanism of action underlies chloroquine's antimicrobial effect against other intracellular pathogens, such as the agents of malaria and tuberculosis. (*J. Clin. Invest.* 1991. 88:351-357.) Key words: ammonium chloride • transferrin • malaria • tuberculosis • iron nitrilotriacetate

Address correspondence to Dr. Thomas F. Byrd, West Los Angeles Veterans Administration Medical Center, Wilshire and Sawtelle, W-111F, Building 500, Room 3221, Los Angeles, CA 90073.

Received for publication 11 September 1990 and in revised form 5 April 1991.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/91/07/0351/07 \$2.00

Volume 88, July 1991, 351-357

Introduction

Legionella pneumophila, the causative agent of Legionnaires' disease, is an intracellular bacterial pathogen which multiplies intracellularly in human mononuclear phagocytes (1). We have previously determined that *L. pneumophila* intracellular multiplication is iron dependent. Our data suggests that *L. pneumophila*, which does not produce iron-chelating siderophores, acquires iron from an intermediate labile iron pool within the mononuclear phagocyte (2). Two major sources of iron for this pool are iron transferrin, the principal supplier of iron for mammalian cells, and ferritin, the major iron storage protein in the cell. Mobilization of iron from both of these sources is dependent upon an acidic environment in endocytic vesicles and lysosomes. Iron-transferrin is endocytized via transferrin receptors and releases ferric ions to the intermediate iron pool only after the endocytic vesicle is acidified (3, 4). Ferritin is thought to recycle iron to the intermediate iron pool by entering lysosomes and undergoing degradation there by pH-dependent proteolysis (5-7).

Chloroquine is a diprotic weak base which raises endocytic and lysosomal pH of eukaryotic cells (8). This action is felt to play an important role in its antimalarial effect (8-10). Not surprisingly, considering that the intracellular transport and mobilization of iron are dependent upon an acidic environment in endocytic vesicles and lysosomes, chloroquine also has been found to interfere with normal iron metabolism in a variety of cell types, including mononuclear phagocytes (11-17).

The dependence of *L. pneumophila* upon intracellular iron and the capacity of chloroquine to interfere with cellular iron metabolism prompted us to hypothesize that chloroquine may inhibit *L. pneumophila* intracellular multiplication by interfering with mononuclear phagocyte iron metabolism and consequently iron availability to intracellular *L. pneumophila*. In this study, we shall show that (a) the lysosomotropic agents chloroquine and ammonium chloride inhibit *L. pneumophila* intracellular multiplication by limiting the availability of iron to the bacterium and (b) this inhibition can be completely reversed by iron nitrilotriacetate, an iron chelate which is uniquely soluble in the neutral or alkaline pH range (18), but not by iron-transferrin which releases iron only at acidic pH (3). Our findings suggest that limitation of iron availability may be a major mechanism for chloroquine's therapeutic effect against intracellular organisms.

Methods

Media and agar. Egg yolk buffer, with or without 1% BSA, and PBS were prepared or obtained as described (1). RPMI 1640 medium with

L-glutamine (RPMI)¹ (Gibco Laboratories, Grand Island, NY) was used in tissue culture experiments. Modified charcoal yeast extract agar (CYEA) was prepared in 100 × 15-mm bacteriologic petri dishes as described (1).

Iron compounds. Ferric nitrilotriacetate (FeNTA) was prepared as previously described (18), and was made fresh for each experiment. Briefly, nitrilotriacetic acid (disodium salt; Sigma Chemical Co., St. Louis, MO) 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. These preparations were filtered through 0.22 micron filter units.

Iron transferrin (Sigma Chemical Co.) was dissolved in RPMI medium and then filtered through a 0.22- μ m filter unit.

Chloroquine and ammonium chloride. Chloroquine (Sigma Chemical Co.) and ammonium chloride (Sigma Chemical Co.) were dissolved in RPMI alone or RPMI with 20 mM Hepes buffer (Sigma Chemical Co.), and filtered through 0.22- μ m filter units.

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°C, as described (1). The bacteria were then passed one time only on CYEA for 80 h, harvested, stored at -70°C, and thawed in a 37°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 as previously described (1).

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 capacity of chloroquine and ammonium chloride to inhibit *L. pneumophila* intracellular multiplication. Freshly explanted monocytes were adhered to Linbro flat-bottomed wells (16 mm in diameter; Flow Laboratories, Inc., McLean, VA) as described (1). The monocyte monolayers, containing approximately 5×10^5 cells, were then incubated in 0.5 ml RPMI containing 20% fresh normal human serum at 37°C in 5% CO₂-95% air. After 24 h, *L. pneumophila* (5×10^3 or 5×10^4 CFU/ml) were added to the monocyte cultures, and the cultures were incubated at 37°C in 5% CO₂-95% air on a gyratory shaker (100 rpm) for 1 h and under stationary conditions thereafter. The infected monocyte cultures were then incubated with chloroquine (1.25–20 μ M) or ammonium chloride (20 mM). In addition, some cultures were incubated with FeNTA (37–75 μ g/ml), NTA (29–58 μ g/ml), or iron transferrin (6 mg/ml). CFU of *L. pneumophila* in each culture were determined daily as described (1). At the end of each experiment, the viability of monocytes was assayed by trypan blue exclusion. All experiments were run in triplicate.

Assay for the effect of chloroquine on *L. pneumophila* multiplication in broth media. Yeast extract broth (YEB) lacking an iron supplement was prepared with 10 g of yeast extract (Difco Laboratories, Detroit, MI), 0.4 g L-cysteine HCl (Fisher Scientific Co.), and 20 g of ACES (Sigma Chemical Co.), adjusted to a pH of 6.9 with 10 N KOH, and filter sterilized. In some cases, the broth was supplemented with ferric pyrophosphate (250 mg/liter) or FeNTA (75 mg/liter). *L. pneumophila* from a stock preparation was grown on CYEA plates, suspended in 70 ml of YEB containing ferric pyrophosphate, and grown to log phase in a 250-ml Erlenmeyer flask. An aliquot of the culture was then removed and seeded into YEB with or without an iron supplement and with or without chloroquine to an initial optical density (OD) of ~ 0.1 at 540

nm (Junior model 35 spectrophotometer; Perkin-Elmer Corp., Oak Brook, IL). The cultures were then incubated at 37°C for 28 h on a rotating platform at 120 rpm, and the OD of the culture media was determined at 4-h intervals.

Assay for the effect of chloroquine on *L. pneumophila* multiplication on agar. CYEA was prepared as usual except that chloroquine, dissolved in PBS, was added to the agar medium to a final concentration of 30 μ M, 150 μ M, 750 μ M, 3.75 mM, and 18.75 mM immediately after the plates were poured. After the agar solidified, the plates were seeded with *L. pneumophila* and the presence or absence of growth on the plates was noted at 5 d.

Results

Chloroquine and ammonium chloride inhibit *L. pneumophila* intracellular multiplication. We first examined if the weak bases chloroquine and ammonium chloride inhibit *L. pneumophila* intracellular multiplication. Monocytes were infected with *L. pneumophila* and then incubated with chloroquine or ammonium chloride. Chloroquine at a 20 μ M concentration and ammonium chloride at a 20 mM concentration both inhibited *L. pneumophila* intracellular multiplication (Fig. 1). With both compounds, the effect was maximal at 2 d. For chloroquine, the mean inhibition in three independent experiments in three subjects was 1.4 ± 0.2 (SEM) logs and for ammonium chloride, the mean inhibition was 1.5 ± 0.2 logs. The effect of these compounds was not due to a toxic effect on the monocyte monolayers, as the monolayers were intact and viable throughout the course of the experiments by trypan blue exclusion.

To determine the effects of lower concentrations of chloroquine, we infected monocytes with *L. pneumophila* and then incubated them without chloroquine, or with chloroquine at concentrations ranging from 1.25 to 20 μ M (Fig. 2). Chloroquine-inhibited *L. pneumophila* intracellular multiplication in a dose-dependent fashion; even at the lowest concentration tested, 1.25 μ M, *L. pneumophila* multiplication was inhibited by 76%. This indicated that chloroquine exerts an inhibitory effect on *L. pneumophila* intracellular multiplication at clinically achievable concentrations, which are in the 1–2 μ M range.

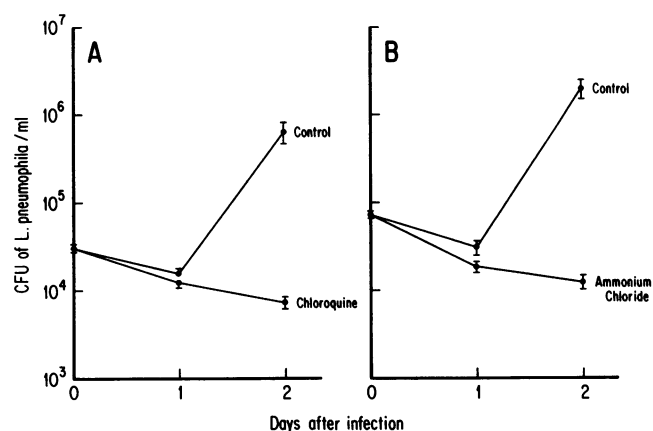


Figure 1. Chloroquine and ammonium chloride inhibit *L. pneumophila* intracellular multiplication in human monocytes. Monocytes in monolayer culture were infected with *L. pneumophila* and then incubated with chloroquine (20 μ M) or control medium (A); or with ammonium chloride (20 mM) or control medium (B). CFU were determined daily. Data are mean \pm SEM for triplicate cultures.

1. Abbreviations used in this paper: ACES, N-[2-acetamido]-2-aminethanesulfonic acid; CYEA, charcoal yeast extract agar; FeNTA, ferric nitrilotriacetate; NTA, sodium nitrilotriacetate; RPMI, RPMI 1640 medium with L-glutamine; YEB, yeast extract broth.

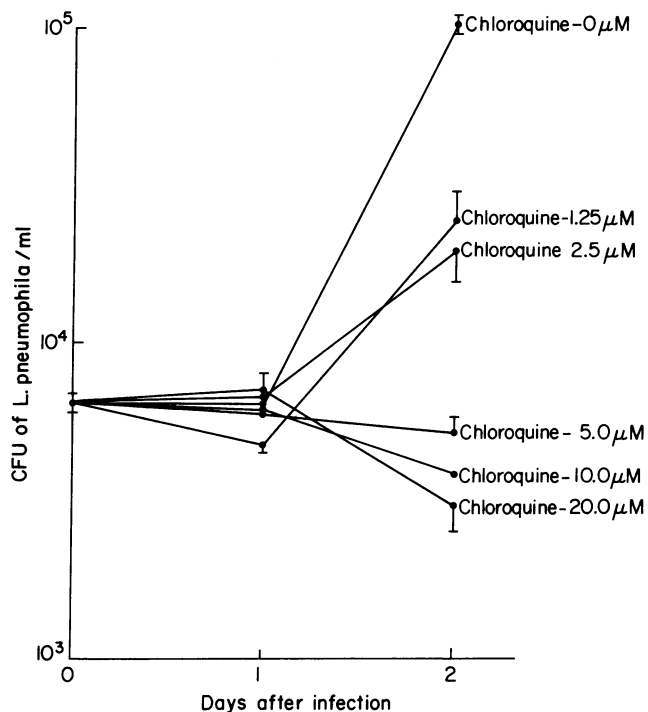


Figure 2. Dose-dependent inhibition of *L. pneumophila* multiplication in human monocytes by chloroquine. Monocytes in monolayer culture were infected with *L. pneumophila* and then incubated with various concentrations of chloroquine ranging from 0 to 20 μM . CFU were determined daily. Data are mean \pm SEM for triplicate cultures.

FeNTA reverses chloroquine and ammonium chloride inhibition of *L. pneumophila* intracellular multiplication. We next investigated if chloroquine and ammonium chloride inhibit *L. pneumophila* multiplication in the mononuclear phagocyte by limiting iron availability. We infected monocytes with *L. pneumophila* in the presence of chloroquine or ammonium chloride, and examined the effects of *FeNTA* on *L. pneumophila* intracellular multiplication. *FeNTA* holds iron in a soluble form at neutral or alkaline pH (18), and thus its solubility would not be affected by changes in intracellular pH induced by chloroquine and ammonium chloride.

FeNTA reversed the inhibitory effect of both chloroquine and ammonium chloride on *L. pneumophila* intracellular multiplication (Fig. 3). In the absence of chloroquine or ammonium chloride, *FeNTA* did not significantly enhance *L. pneumophila* multiplication indicating that iron was not limiting to *L. pneumophila* under the conditions of the experiment.

To confirm that the effect of *FeNTA* was due to iron and not to nitrilotriacetate, we compared the effect of *FeNTA* with that of NTA on the capacity of chloroquine and ammonium chloride to inhibit *L. pneumophila* multiplication. Whereas *FeNTA* completely reversed the inhibitory effect of chloroquine and ammonium chloride, NTA had no effect (Fig. 4). Analysis by trypan blue exclusion showed that NTA-treated monolayers were intact and viable throughout the course of the experiment.

These results indicated that the inhibitory effect of chloroquine and ammonium chloride on *L. pneumophila* multiplication was due to alterations in iron metabolism in the mononuclear phagocyte.

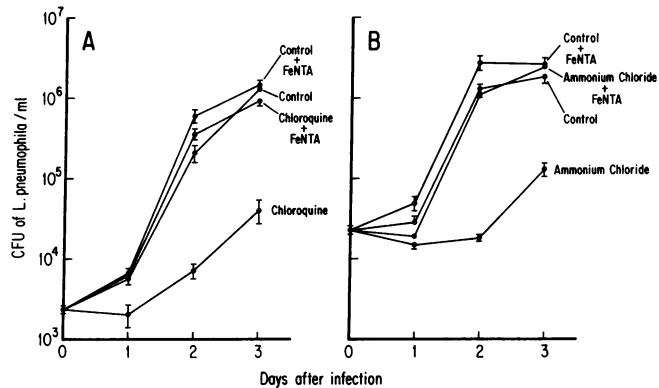


Figure 3. *FeNTA* reverses the inhibitory effect of chloroquine and ammonium chloride on *L. pneumophila* intracellular multiplication in human monocytes. Monocytes in monolayer culture were infected with *L. pneumophila* and then incubated with chloroquine (20 μM) or control medium in the presence or absence of *FeNTA* (75 $\mu\text{g}/\text{ml}$) (A); or with ammonium chloride (20 mM) or control medium in the presence or absence of *FeNTA* (75 $\mu\text{g}/\text{ml}$) (B). CFU were determined daily. Data are mean \pm SEM for triplicate cultures.

FeNTA, but not iron-transferrin, reverses the capacity of chloroquine and ammonium chloride to inhibit *L. pneumophila* intracellular multiplication. We have previously shown that iron transferrin is capable of entering the mononuclear phagocyte and supplying iron to intracellular *L. pneumophila* (2). However, both chloroquine and ammonium chloride have been found to prevent the accumulation of iron in cells incubated with iron transferrin (11–17). By increasing endocytic vesicle pH, these agents prevent the unloading of iron from endocytized transferrin, a process which is dependent on endocytic vesicle acidification (19). In addition, ammonium chloride at concentrations > 5 mM has been reported to inhibit

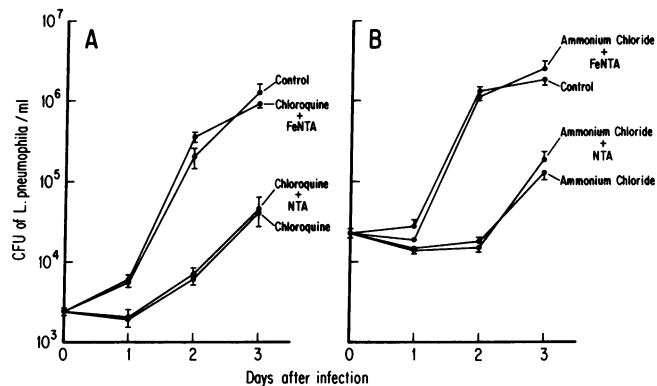


Figure 4. *FeNTA* but not NTA reverses the inhibitory effect of chloroquine and ammonium chloride on *L. pneumophila* intracellular multiplication in human monocytes. Monocytes in monolayer culture were infected with *L. pneumophila* and then incubated with chloroquine (20 μM), control medium, chloroquine plus *FeNTA* (75 $\mu\text{g}/\text{ml}$) or chloroquine plus NTA (58 $\mu\text{g}/\text{ml}$) (equivalent amounts of nitrilotriacetate) (A); or with ammonium chloride (20 mM), control medium, ammonium chloride plus *FeNTA* (75 $\mu\text{g}/\text{ml}$) or ammonium chloride plus NTA (58 $\mu\text{g}/\text{ml}$) (equivalent amounts of nitrilotriacetate) (B). CFU were determined daily. Data are mean \pm SEM for triplicate cultures.

transferrin binding to cell surface transferrin receptors (12). This suggested that iron-transferrin, the major physiologic source of iron to the mammalian cell, may not be capable of reversing the inhibitory effect of chloroquine on *L. pneumophila* intracellular multiplication. To test this hypothesis, we compared the effects of FeNTA with that of iron-transferrin on chloroquine and ammonium chloride-induced inhibition of *L. pneumophila* intracellular multiplication.

Whereas FeNTA reversed chloroquine and ammonium chloride-induced inhibition of *L. pneumophila* multiplication, iron-transferrin had no effect (Fig. 5). This provided further evidence that chloroquine and ammonium chloride interfere with intracellular iron metabolism via their effects on the pH of intracellular compartments in mononuclear phagocytes.

Chloroquine does not inhibit L. pneumophila multiplication in broth medium except at extremely high concentrations, and inhibition at these levels is not reversed by iron. The capacity of FeNTA to reverse the inhibitory effect of chloroquine on *L. pneumophila* intracellular multiplication indicated that chloroquine was not directly toxic to *L. pneumophila* in monocytes. To examine further for potential direct effects of chloroquine on *L. pneumophila*, we investigated the effect of chloroquine on *L. pneumophila* multiplication in broth culture, using concentrations of chloroquine ranging up to 15,000 times the lowest concentration used in the tissue culture experiments (1.25 μ M) described above.

In the absence of supplemental iron, chloroquine had no effect on *L. pneumophila* multiplication at concentrations of 30, 150, or 750 μ M (24, 120, and 600 times the lowest effective concentration in tissue culture experiments). At a concentration of 3.75 mM (3,000 times the lowest effective concentration tested in tissue culture), chloroquine had a slight effect on *L. pneumophila* multiplication, and at a concentration of 18.75 mM (15,000 times the lowest effective concentration tested in tissue culture), chloroquine markedly inhibited *L. pneumophila* multiplication (Fig. 6). The slight effect of 3.75 mM chloroquine and the major effect of 18.75 mM chloro-

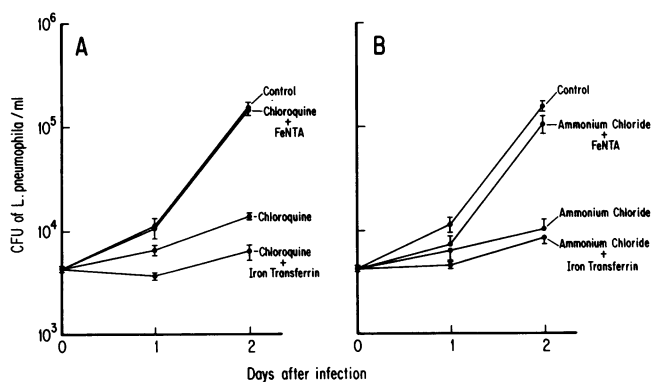


Figure 5. FeNTA but not iron-transferrin reverses the inhibitory effect of chloroquine and ammonium chloride on *L. pneumophila* multiplication in human monocytes. Monocytes in monolayer culture were infected with *L. pneumophila* and then incubated with chloroquine (20 μ M), control medium, chloroquine plus FeNTA (37 μ g/ml) or chloroquine plus iron-transferrin (6 mg/ml) (equivalent amounts of iron) (A); or with ammonium chloride (20 mM), control medium, ammonium chloride plus FeNTA (37 μ g/ml), or ammonium chloride plus iron-transferrin (6 mg/ml) (equivalent amounts of iron). CFU were determined daily. Data are mean \pm SEM for triplicate cultures.

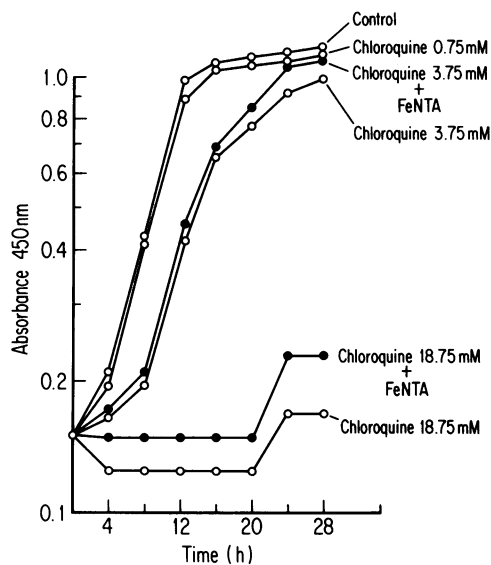


Figure 6. Chloroquine does not inhibit *L. pneumophila* multiplication in broth media except at very high concentrations, and inhibition at these levels is not reversed by iron. *L. pneumophila* was cultured in YEB containing chloroquine at concentrations ranging from 0 to 18.75 mM in the presence or absence of FeNTA (75 μ g/ml). OD of the broth was determined every 4 h.

quine on *L. pneumophila* multiplication was not reversed by FeNTA (Fig. 6), indicating that the effect of these extremely high concentrations of chloroquine was not on iron availability.

Similarly, chloroquine inhibited *L. pneumophila* multiplication on CYEA only at a concentration of 18.75 mM, 15,000 times the lowest effective concentration tested in tissue culture experiments.

Discussion

This paper demonstrates that chloroquine and ammonium chloride inhibit the intracellular multiplication of *L. pneumophila* in human monocytes by interfering with iron acquisition by the bacterium. Chloroquine and ammonium chloride are structurally unrelated, but both compounds are weak bases. Consistent with this, their common inhibitory effect on *L. pneumophila* intracellular multiplication appears secondary to a shared capacity to raise endocytic vesicle and lysosomal pH and consequently to interfere with iron availability, rather than from a direct toxic effect on *L. pneumophila*.

By increasing lysosomal and endocytic vesicle pH, chloroquine and ammonium chloride may interfere with intracellular iron metabolism in at least two major ways. First, these compounds may inhibit the pH-dependent release of iron from iron-saturated transferrin endocytized via transferrin receptors. After the transferrin receptor binds diferric transferrin, the transferrin receptor-transferrin complex is endocytized, and the endocytic vesicle is acidified. As the pH decreases below 7.0, ferric ions dissociate from transferrin and are distributed intracellularly. As the pH drops from 7.0 to 6.0, one of the two iron molecules bound to transferrin dissociates so that at pH 6.0, transferrin is \sim 50% saturated. As the pH drops from 6.0 to 5.0, the other iron molecule bound to transferrin dissociates so that at pH 5.0, transferrin is unsaturated (20, 21). The apo-

transferrin remains bound to the transferrin receptor and is recycled to the cell surface (3).

At concentrations similar to those used in this study, chloroquine and ammonium chloride have been found to raise the pH of acid intravesicular compartments including coated vesicles, endosomes, lysosomes, and the Golgi complex (8). In mouse fibroblasts and peritoneal macrophages, these compounds raise the endocytic and lysosomal pH from ~ 5.0 to 6.2 (19, 22). At this pH level, chloroquine and ammonium chloride would inhibit the release of > 50% of the iron from transferrin. Consistent with this, chloroquine and ammonium chloride have been found to reduce markedly the release of iron from endocytized diferric transferrin in a variety of cell types, including mononuclear phagocytes, without affecting receptor recycling (11–17).

The second way that chloroquine and ammonium chloride may interfere with intracellular iron metabolism is by blocking recycling of iron from ferritin. Recent studies have suggested that iron bound to ferritin is released to the intermediate labile iron pool of the cell after ferritin is degraded in lysosomes. By raising intralysosomal pH, chloroquine and ammonium chloride may block degradation of ferritin by acid proteases (5–7).

In previous studies, we have found that intracellular *L. pneumophila* utilizes iron derived from both iron-transferrin and ferritin (2, 23). Thus, by interfering with the release of iron from these two major sources to the intermediate labile iron pool of the mononuclear phagocyte, chloroquine and ammonium chloride may decrease the availability of iron to intracellular *L. pneumophila* and inhibit multiplication of this intracellular pathogen. Consistent with this hypothesis, FeNTA reverses the capacity of chloroquine and ammonium chloride to inhibit *L. pneumophila* multiplication, and its ability to do so is dependent upon its iron content, as NTA has no effect. We have previously demonstrated that FeNTA can enter the monocyte and supply iron to intracellular *L. pneumophila* (24). FeNTA is unique among nonphysiologic iron chelates in that it holds iron in a soluble form in the neutral to alkaline pH range (18). Whereas, in the mildly acidic to alkaline pH range, ferric salts, such as ferric nitrate and ferric chloride, form hydrolytic polymers from which iron is relatively inaccessible, FeNTA exists predominantly in a monomeric form from which iron is readily accessible. For example, at a pH of 7.4, ~ 85% of FeNTA exists in a monomeric form, most of the remainder exists as a dimer, and only a small proportion exists as a polymeric complex (18). Thus, the increase in intravesicular pH induced by chloroquine and ammonium chloride in monocytes infected with *L. pneumophila* should not inhibit the capacity of FeNTA to deliver iron to the bacterium. In contrast to FeNTA, iron-transferrin, which releases iron only at acidic pH (3), is not able to reverse the inhibitory effect of chloroquine and ammonium chloride.

As with monocytes treated with chloroquine and ammonium chloride, interferon gamma-activated monocytes inhibit *L. pneumophila* intracellular multiplication, and they do so by limiting the availability of intracellular iron (2). Also as with monocytes treated with chloroquine and ammonium chloride, interferon gamma-activated monocytes reduce iron acquisition from iron-transferrin and ferritin. However, the interferon gamma-activated monocytes appear to do so at different points in the metabolic pathways of these iron proteins than do monocytes treated with the lysosomotropic agents. In the case of iron-transferrin, the interferon gamma-activated monocyte

markedly downregulates transferrin receptors on the cell surface (2), thereby reducing the uptake of iron via the transferrin receptor-transferrin endocytic pathway, the major conduit for iron into the cell. In the case of ferritin, the interferon gamma-activated monocyte markedly decreases the intracellular concentration of this iron storage protein (23), thereby decreasing the amount of iron available from the major iron storage pool of the cell. Thus, it appears that the net effect of interferon gamma is to reduce the iron stores of the cell, whereas the net effect of chloroquine and ammonium chloride is to reduce the release of iron from intracellular pools.

While chloroquine had no effect on *L. pneumophila* multiplication in broth or on agar at low doses, it inhibited *L. pneumophila* multiplication at very high doses. A major effect was noted only at a concentration of chloroquine ~ 15,000-fold the lowest effective dose tested in the tissue culture studies. The inhibitory effect of high dose chloroquine on *L. pneumophila* multiplication in broth culture was not reversed by iron, suggesting another mechanism of action was operative at high chloroquine concentrations. At concentrations of 1 mM, chloroquine has been found to inhibit the multiplication of a number of bacteria in broth culture, reportedly by interfering with DNA replication (25). Such a mechanism may account for the inhibitory effect of high concentrations of chloroquine on *L. pneumophila* multiplication in artificial media.

Chloroquine has been the mainstay of treatment and chemoprophylaxis of malaria for over four decades. Chloroquine has been found to raise the pH of the malaria parasite's food vacuole, which is analogous to secondary lysosomes in mammalian cells (8, 9). It has been postulated that as a result of this, chloroquine interferes with the digestion of hemoglobin in the food vacuole, which provides the parasite with essential amino acids (26). However, as in the case of *L. pneumophila*, intracellular growth of the malaria parasite is iron-dependent (27). Multiplication of the malaria parasite in erythrocytes is inhibited by the same concentration of deferoxamine that inhibits multiplication of *L. pneumophila* in human monocytes (2, 27). The source of this iron for the malaria parasite is unclear. Some evidence suggests that it derives from host iron transferrin (28–30) and other evidence suggests that it derives from host erythrocyte hemoglobin (31, 32). Whatever the source of iron, its intracellular mobilization is likely dependent upon acidic conditions. The pH of the malaria parasite's food vacuole has been reported to be in the range of 5.2–5.4. Both chloroquine and ammonium chloride have been found to increase the pH of the food vacuole by up to 1.0 pH unit depending upon the extracellular concentration of these drugs. At concentrations which increase the pH of the food vacuole, these drugs begin to inhibit *P. falciparum* growth (10). It is therefore conceivable that chloroquine exerts an antiparasitic effect by decreasing iron availability to the malaria parasite, as a consequence of its effect on intracellular pH.

The concentration of chloroquine required to raise the pH of vesicles of chloroquine-sensitive malaria parasites is in the 1–20 nM range, substantially lower than that required to raise the pH of acid vesicles in mammalian cells (10). The malaria parasite has a chloroquine-concentrating mechanism that results in a 1,000-fold higher concentration of drug in parasite acid vesicles than predicted on the basis of chloroquine's properties as a weak base. In contrast, the accumulation of chloroquine in mammalian cell acid vesicles is consistent with its properties as a weak base (8–10). Paralleling these differences,

the concentrations of chloroquine that inhibit the growth of chloroquine-sensitive malaria parasites, in the same range that raise parasite intravesicular pH, are substantially lower than those which inhibit the intracellular multiplication of *L. pneumophila* in human monocytes (10). Thus, in the case of both pathogens, the concentration of chloroquine required to inhibit growth is equivalent to that required to raise intravesicular pH in malaria parasites or monocyte hosts of *L. pneumophila*.

Consistent with the idea that chloroquine's antiparasitic effect is mediated by its effects on intravesicular pH, the concentration of chloroquine required to inhibit chloroquine-resistant strains of malaria, which have a diminished chloroquine concentrating capacity, is much higher and in the range of that required to raise the pH of mammalian cells. For example, in one study, chloroquine at a concentration of 1–10 nM was required to raise the pH and inhibit a chloroquine-sensitive strain of malaria, whereas 0.1–1 μ M chloroquine was required to raise the pH and inhibit a chloroquine-resistant strain (10). Furthermore, the concentration of ammonium chloride required to inhibit both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*, neither of which are known to have a mechanism for concentrating this weak base, is in the 10 mM range, a concentration equivalent to that required to raise intravesicular pH in mammalian cells (10, 33).

In addition to its well known effect on the malaria parasite, chloroquine has been reported to inhibit the intracellular multiplication of *Mycobacterium tuberculosis* in both human monocyte-derived macrophages and mouse peritoneal macrophages at the same concentration used in our study (34, 35). The mechanism of this inhibitory effect has not been defined, but it appears to be due to an effect of chloroquine on macrophage metabolism rather than to a direct effect on the mycobacterium. In support of this idea, chloroquine inhibited the multiplication of two strains of *M. tuberculosis* H37Rv in cultured mouse macrophages to the same degree, although the two strains exhibited markedly different susceptibility to chloroquine in broth media (34). It seems possible that chloroquine inhibits *M. tuberculosis* intracellular multiplication by raising intracellular pH and limiting the availability of iron to this bacterium as it does to *L. pneumophila*.

As noted above, chloroquine and interferon gamma inhibit *L. pneumophila* intracellular multiplication by interfering with monocyte iron metabolism in different ways, the former by decreasing the intracellular release of iron and the latter by decreasing intracellular iron pools. Acting still differently, by chelating iron in the intermediate labile iron pool of the cell, deferoxamine also inhibits *L. pneumophila* intracellular multiplication (2). Particularly in view of their disparate effects on iron metabolism, these three compounds may act additively or synergistically in vivo to inhibit the multiplication of *L. pneumophila* and potentially other intracellular pathogens. Thus, a therapeutic role for one or more combinations of these drugs in the treatment of infections caused by intracellular pathogens is conceivable.

The lowest concentration of chloroquine that inhibited *L. pneumophila* intracellular multiplication in this study, 1.25 μ M, the lowest concentration tested, is clinically achievable in humans. Serum concentrations of 1–2 μ M have been achieved with dosage regimens of chloroquine used to treat rheumatoid arthritis (36, 37). In summary, our study shows that chloroquine inhibits the intracellular multiplication of *L. pneumo-*

phila by interfering with intracellular iron metabolism. Possibly, the same mechanism of action underlies chloroquine's antimicrobial effect on malaria parasites and the tubercle bacillus.

Acknowledgments

We are grateful to Ms. Barbara Jane Dillon and Mr. Chai Chaloyphian for expert technical assistance with experiments.

This work was supported by grants AI-22421 and AI-28825 from the National Institutes of Health. Dr. Horwitz is Gordon MacDonald Scholar at University of California, Los Angeles, and recipient of a Faculty Research Award from the American Cancer Society. During the time this work was performed, Dr. Byrd was a recipient of a National Research Service Award from the National Institutes of Health.

References

- Horwitz, M. A., and S. C. Silverstein. 1980. The Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. *J. Clin. Invest.* 66:441–450.
- Byrd, T. F., and M. A. Horwitz. 1989. Interferon gamma-activated human monocytes downregulate transferrin receptors and inhibit the intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron. *J. Clin. Invest.* 83:1457–1465.
- Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 80:2258–2262.
- Jacobs, A. 1977. Low molecular weight intracellular iron transport compounds. *Blood.* 50:433–439.
- Laub, R., Y. Schneider, J. Octave, A. Trouet, and R. R. Crichton. 1985. Cellular pharmacology of deferoxamine B and derivatives in cultured rat hepatocytes in relation to iron mobilization. *Biochem. Pharmacol.* 34:1175–1183.
- Starke, P. E., J. D. Gilbertson, and J. L. Farber. 1985. Lysosomal origin of the ferric iron required for cell killing by hydrogen peroxide. *Biochem. Biophys. Res. Commun.* 133:371–379.
- Sibille, J., H. Kondo, and P. Aisen. 1989. Uptake of ferritin and iron bound to ferritin by rat hepatocytes: modulation by apotransferrin, iron chelators and chloroquine. *Biochim. Biophys. Acta.* 1010:204–208.
- Krogstad, D. J., and P. H. Schlesinger. 1987. Acid-vesicle function, intracellular pathogens, and the action of chloroquine against *Plasmodium falciparum*. *N. Engl. J. Med.* 317:542–549.
- Krogstad, D. J., and P. H. Schlesinger. 1986. A perspective on antimalarial action: effects of weak bases on *Plasmodium falciparum*. *Biochem. Pharmacol.* 35:547–552.
- Krogstad, D. J., P. H. Schlesinger, and I. Y. Gluzman. 1985. Antimalarials increase vesicle pH in *Plasmodium falciparum*. *J. Cell Biol.* 101:2302–2309.
- Swainman, K. F., and V. L. Machen. 1986. Chloroquine reduces neuronal and glial iron uptake. *J. Neurochem.* 46:652–654.
- Forsbeck, K., and K. Nilsson. 1983. Iron metabolism of established human hematopoietic cell lines in vitro. *Exp. Cell Res.* 144:3223–3332.
- Iacopetta, B. J., and E. H. Morgan. 1983. The kinetics of transferrin endocytosis and iron uptake from transferrin in rabbit reticulocytes. *J. Biol. Chem.* 258:9108–9115.
- Octave, J. N., Y. J. Schneider, P. Hoffman, A. Troluet, and R. R. Crichton. 1979. Transferrin protein and iron uptake by cultured rat fibroblasts. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 108:127–130.
- Karin, M., and M. Mintz. 1981. Receptor-mediated endocytosis of transferrin in developmentally totipotent mouse teratocarcinoma stem cells. *J. Biol. Chem.* 256:3245–3252.
- Armstrong, N. J., and E. H. Morgan. 1983. The effect of lysosomotropic bases and inhibitors of transglutaminase on iron uptake by immature erythroid cells. *Biochim. Biophys. Acta.* 762:175–186.
- Baynes, R., G. Bukofzer, T. Bothwell, W. Bezwoda, and B. Macfarlane. 1987. Transferrin receptors and transferrin iron uptake by cultured human blood monocytes. *Eur. J. Cell Biol.* 43:372–376.
- Bates, G. W., and J. Wernicke. 1971. The kinetics and mechanism of iron (III) exchange between chelates and transferrin. *J. Biol. Chem.* 246:3679–3686.
- Maxfield, F. R. 1982. Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. *J. Cell Biol.* 95:676–681.
- Princiotta, J. V., and E. J. Zapolski. 1975. Difference between the two iron-binding sites of transferrin. *Nature (Lond.)* 255:87–88.
- Lestas, A. N. 1976. The effect of pH upon human transferrin: selective labeling of the two iron-binding sites. *Br. J. Haematol.* 32:341–350.

22. Poole, B., and S. Ohkuma. 1981. Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. *J. Cell Biol.* 90:665-669.
23. Byrd, T. F., and M. A. Horwitz. 1990. Interferon-gamma activated human monocytes downregulate the intracellular concentration of ferritin: a potential new mechanism for limiting iron availability to *Legionella pneumophila* and subsequently inhibiting intracellular multiplication. *Clin. Res.* 38:481A. (Abstr.)
24. Byrd, T. F., and M. A. Horwitz. 1991. 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 non-physiologic iron chelates reverse monocyte activation against *Legionella pneumophila*. *J. Clin. Invest.* In press.
25. Ciak, J., and F. E. Hahn. 1966. Chloroquine: mode of action. *Science (Wash. DC)*. 151:347-349.
26. Zahng, Y. 1987. Inhibition of hemoglobin degradation in *Plasmodium falciparum* by chloroquine and ammonium chloride. *Exp. Parasitol.* 64:322-327.
27. Raventos-Suarez, C., Pollack, S., and R. L. Nagel. 1982. *Plasmodium falciparum*: inhibition of in vitro growth by desferrioxamine. *Am. J. Trop. Med. Hyg.* 31:919-922.
28. Pollack, S., and J. Fleming. 1984. *Plasmodium falciparum* takes up iron from transferrin. *Br. J. Haematol.* 58:289-293.
29. Haldar, K., C. L. Henderson, and G. A. M. Cross. 1986. Identification of the parasite transferrin receptor of *Plasmodium falciparum*-infected erythrocytes and its acylation via 1,2-diacyl-sn-glycerol. *Proc. Natl. Acad. Sci. USA.* 83:8565-8569.
30. Rodriguez, M. H., and M. Jungery. 1986. A protein on *Plasmodium falciparum*-infected erythrocytes functions as a transferrin receptor. *Nature (Lond.)*. 324:308-391.
31. Hershko, C., and T. E. A. Peto. 1988. Deferoxamine inhibition of malaria is independent of host iron status. *J. Exp. Med.* 168:375-387.
32. Peto, T. E. A., and J. L. Thompson. 1986. A reappraisal of the effects of iron and desferrioxamine on the growth of *Plasmodium falciparum* in vitro: the unimportance of serum iron. *Br. J. Haematol.* 63:273-280.
33. Krogstad, D. J., and P. H. Schlesinger. 1987. The basis of antimalarial action: non-weak base effects of chloroquine on acid vesicle pH. *Am. J. Trop. Med. Hyg.* 36:213-220.
34. Hart, P. D. 1982. Evidence that killing of *Mycobacterium tuberculosis* by chloroquine-treated culture macrophages is cell-mediated and not direct. *J. Med. Microbiol.* 15:iv. (Abstr.)
35. Crowle, A. J., and M. H. May. 1989. Chloroquine, a potentially new antituberculous drug. In Program and Abstracts of the 29th ICAAC. 29:50. (Abstr.)
36. MacKenzie, A. H. 1983. Pharmacologic actions of 4-aminoquinoline compounds. *Am. J. Med.* 75 (Suppl.):5-10.
37. Wollheim, F. A., A. Hanson, and C. Laurell. 1978. Chloroquine treatment in rheumatoid arthritis. *Scand. J. Rheum.* 7:171-176.