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Intracellular Multiplication of Legionnaires' Disease Bacteria (*Legionella pneumophila*) in Human Monocytes is Reversibly Inhibited by Erythromycin and Rifampin

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ABSTRACT We have previously reported that virulent egg yolk-grown *Legionella pneumophila*, Philadelphia 1 strain, multiplies intracellularly in human blood monocytes and only intracellularly under tissue culture conditions. In this paper, we have investigated the effect of erythromycin and rifampin on *L. pneumophila*-monocyte interaction in vitro; erythromycin and rifampin are currently the drugs of choice for the treatment of Legionnaires' disease.

The intracellular multiplication of *L. pneumophila* is inhibited by erythromycin and rifampin, as measured by colony-forming units, whether the antibiotics are added just before or just after infection of monocytes with *L. pneumophila*, or 2 d after infection when *L. pneumophila* is in the logarithmic phase of growth in monocytes. Intracellular multiplication of *L. pneumophila* is inhibited by 1.25 μg/ml but not ≤0.125 μg/ml erythromycin and 0.01 μg/ml but not ≤0.001 μg/ml rifampin. These concentrations of antibiotics are comparable to those that inhibit extracellular multiplication of *L. pneumophila* under cell-free conditions in artificial medium; the minimal inhibitory concentration is 0.37 μg/ml for erythromycin and 0.002 μg/ml for rifampin. Multiplication of *L. pneumophila* in the logarithmic phase of growth in monocytes is inhibited within 1 h of the addition of antibiotics. Intracellular bacteria inhibited from multiplying by antibiotics are not killed. By electron microscopy, the bacteria appear intact within membrane-bound vacuoles, studded with ribosomelike structures.

L. pneumophila multiplying extracellularly on artificial medium is killed readily by relatively low concentrations of erythromycin and rifampin; the minimal bactericidal concentration is 1 μg/ml for erythromycin and 0.009 μg/ml for rifampin. In contrast, *L. pneumophila* multiplying intracellularly is resistant to killing by these concentrations of erythromycin and rifampin or by concentrations equal to or greater than peak serum levels in humans. Extracellular *L. pneumophila* in stationary phase is also resistant to killing by erythromycin and rifampin. These findings, taken together with our previous work, indicate that, in vivo, *L. pneumophila* is resistant to killing by erythromycin and rifampin.

Inhibition of *L. pneumophila* multiplication in monocytes by antibiotics is reversible; when the antibiotics are removed from infected monocyte cultures after 2 d, *L. pneumophila* resumes multiplication.

This study indicates that patients with Legionnaires' disease under treatment with erythromycin and rifampin require host defenses to eliminate *L. pneumophila*, and that inadequate host defenses may result in relapse after cessation of therapy.

INTRODUCTION

We have previously reported that *Legionella pneumophila*, the agent of Legionnaires' disease, is a facultative intracellular pathogen. The bacteria multiply intracellularly in human monocytes and alveolar macrophages, and, under tissue culture conditions, multiplication is exclusively intracellular (1, 2). Virulent in vivo grown *L. pneumophila* is completely resistant to the bactericidal effects of human serum, even in the presence of high titer anti-*L. pneumophila* antibody (3). The bacteria also resist killing by human polymorphonuclear leukocytes (PMN) even in the presence of specific antibody and complement (3, 4). Antibody and complement promote the binding of *L. pneumophila* to monocytes, but do not inhibit the rate of bacterial multiplication in monocytes (4). In vitro ac-

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tivated human monocytes and alveolar macrophages do inhibit the intracellular multiplication of *L. pneumophila* (2, 5).

Erythromycin and rifampin are currently the drugs of choice for the treatment of Legionnaires' disease. Both clinical experience and in vivo studies indicate that these antibiotics are efficacious in treating Legionnaires' disease (6–9). *L. pneumophila* grown under cell-free conditions on artificial media is inhibited from multiplying by these antibiotics and is killed by concentrations of these antibiotics that are the same or somewhat higher than the minimal inhibitory concentration (MIC)[1] (10, 11).

In this study, we have examined the effect of erythromycin and rifampin on *L. pneumophila* located intracellularly in human monocytes. We shall demonstrate (*a*) that these antibiotics rapidly inhibit the intracellular multiplication of *L. pneumophila* at concentrations comparable to those that inhibit *L. pneumophila* multiplication under cell-free conditions in artificial medium; (*b*) that *L. pneumophila* multiplying in human monocytes is resistant to killing by concentrations of erythromycin and rifampin that are bactericidal to *L. pneumophila* multiplying extracellularly in artificial medium; (*c*) that *L. pneumophila* multiplying in human monocytes is resistant to killing by erythromycin and rifampin even at concentrations of these antibiotics comparable to or higher than peak blood levels in humans; and (*d*) that *L. pneumophila* inhibited from multiplying in monocytes by erythromycin and rifampin retains its capacity to multiply in monocytes after the antibiotics are removed from the medium.

METHODS

Media. Egg yolk buffer (EYB), with (EYB-BSA) or without 1% bovine serum albumin, and RPMI 1640 medium were prepared or obtained as described previously (1). Buffered yeast extract (BYE) broth, pH 6.9, was prepared with 5 g/liter bovine serum albumin fraction V (Reheis Co., Inc., Armour Pharmaceutical Co., Phoenix, AZ), 10 g/liter yeast extract (Difco Laboratories, Detroit, MI), 0.25 g/liter ferric pyrophosphate soluble (Center for Disease Control, Atlanta, GA), 0.4 g/liter L-cysteine hydrochloride · H_2O (Fisher Scientific Co., Pittsburgh, PA), and 10 g/liter *N*-[2-acetamido]-2-aminoethane sulfonic acid (ACES) (Sigma Chemical Co., St. Louis, MO) essentially as described (12) but with bovine serum albumin instead of α-ketoglutarate.[2]

Antibiotics. Erythromycin base suitable for antimicrobial susceptibility testing (Abbott Diagnostics, Diagnostic Products, North Chicago, IL), 980 μg activity/mg, was dissolved in methanol, diluted in water to 1,000 μg activity/ml, and stored in small aliquots in vials at −70°C. On the day of the experiments the frozen preparation was thawed and diluted to the desired concentration in RPMI 1640 medium. Rifampin (Rimactane diagnostic powder, Ciba Pharmaceutical Company, Summit, NJ), 1,014 μg activity/mg was stored under vacuum at 4°C. On the day of the experiment, the powder was dissolved in methanol and diluted to the desired concentration first in water and then in RPMI 1640 medium. In this paper, the concentrations of antibiotics are stated in micrograms per milliliter, but refer to micrograms of antibiotic activity per milliliter.

Agar. Modified charcoal yeast extract agar was prepared in 100×15-mm petri dishes (1).

Serum. Venous blood was obtained, clotted, and serum separated and stored at −70°C until use (13). Normal (nonimmune) human serum (type AB) with an indirect fluorescent antibody anti-*L. pneumophila* titer (14) of <1:64 was obtained from an adult donor not known tc have ever had Legionnaires' disease.

Bacteria. L. pneumophila, Philadelphia 1 strain, was grown in embryonated hens' eggs, harvested, tested for viability and for the presence of contaminating bacteria, stored at −70°C, and partially purified by differential centrifugation just before use (1).

Human blood mononuclear cells. Mononuclear cells used in experiments with *L. pneumophila* were obtained from the blood of a normal adult donor not known to have ever had Legionnaires' disease and with an indirect fluorescent anti-*L. pneumophila* antibody titer of <1:64. The mononuclear cell fraction was obtained by centrifugation over a Ficoll-sodium diatrizoate solution (1); the cells were >99% viable by trypan blue exclusion. Examination of a stained cytocentrifuged sample revealed that the mononuclear cell fraction contained ~40% monocytes, 58.5% lymphocytes, and 1.5% PMN. The adherent subpopulation (containing >90% monocytes) was prepared as described (1).

Assay for effect of antibiotics on L. pneumophila multiplication in monocytes. L. pneumophila (1 or 5×10^3 colony-forming units [CFU]/ml) were incubated in 17 × 100-mm plastic tubes (2057, Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, CA) in 5% CO_2–95% air with 5×10^6 mononuclear cells in 2 ml RPMI medium containing 15% fresh human serum and either erythromycin in concentrations ranging from 0 to 1.25 μg/ml or rifampin in concentrations ranging from 0 to 0.01 μg/ml. The cultures were incubated on a gyratory shaker (100 rpm) for 1 h and under stationary conditions thereafter. CFU of *L. pneumophila* in each culture were determined daily (1). In some experiments, erythromycin and rifampin were added to cultures immediately after shaking instead of before shaking. In other experiments, antibiotics were not added until 48 h after infection when *L. pneumophila* were in the logarithmic phase of multiplication in monocytes.

Assay for determining the MIC and minimal bactericidal concentration (MBC) of erythromycin and rifampin for L. pneumophila multiplying extracellularly. The MIC and MBC were determined by modifications of standard methodology (15, 16). *L. pneumophila* was grown in BYE broth at 37°C on a rotatory shaker to midlogarithmic phase (OD of 0.400 at 540 nm measured in a Coleman 44 model spectrophotometer [Perkin-Elmer Corp., Norwalk, CT]). The culture was then diluted in BYE broth to two times the concentration to be tested; in each experiment, a relatively high and relatively low concentration of bacteria in the range 7.5×10^4 to 2.5×10^7 CFU/ml were tested. 1 ml of each concentration of bacteria to be tested was added to plastic tubes

[1] *Abbreviations used in this paper*: BYE, buffered yeast extract broth; CFU, colony-forming units; EYB, egg yolk buffer; EYB-BSA, EYB containing 1% bovine serum albumin; MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration.

[2] Feeley, J. C. Personal communication.

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containing 1 ml of twofold dilutions of antibiotic in BYE broth, such that the final concentration of antibiotic ranged from 0.0312 to 16 μg/ml erythromycin and 0.000125 to 1 μg/ml rifampin. Control tubes contained no antibiotic. Each concentration of bacteria and antibiotic were tested in triplicate. The tubes were sealed and placed horizontally on a Nutator (Clay-Adams Div., Becton, Dickinson & Co.) and rotated continuously at 37°C for 72 h.

To determine the MIC, the tubes were examined for macroscopic growth at 24, 48, and 72 h by visual inspection and by measuring the optical density spectrophotometrically. The MIC was the lowest concentration of antibiotic inhibiting macroscopic growth. The results for 24, 48, and 72 h were the same or within one twofold dilution of each other in all experiments; only the 48-h value is stated in this paper.

To determine the MBC, tubes with antibiotic concentrations equal to or greater than MIC at 48 h were assayed for *L. pneumophila*; samples of each tube's contents were serially diluted in BYE broth and 100 μl of appropriate dilutions spread onto charcoal yeast extract agar plates to determine CFU per milliliter as described (1). The mean CFU per milliliter for three replicate tubes for each concentration of bacteria and antibiotic was determined. The MBC was the lowest concentration of antibiotic that resulted in ≥3 log mean decrease in CFU per milliliter from the initial concentration (16).

Assay for the effect of preincubation of monocytes with antibiotics on L. pneumophila multiplication in monocytes. Mononuclear cells (5×10^6) in 2 ml RPMI 1640 medium containing 15% fresh human serum were incubated in 17 × 100-mm plastic tubes at 37°C in 5% CO_2–95% air for 48 h with erythromycin (1.25 μg/ml), rifampin (0.01 μg/ml), or without antibiotics. After 48 h, antibiotics were removed by washing leukocytes in all cultures by centrifugation at 200 *g* for 10 min. The leukocyte pellets were resuspended in 2 ml RPMI 1640 medium containing 15% fresh human serum and incubated with or without antibiotics as follows. Replicate cultures initially containing erythromycin were split into two groups; one group was again incubated with erythromycin (1.25 μg/ml) and the other group was incubated without any antibiotic. Similarly, replicate cultures initially containing rifampin were split into two groups; one group was incubated with rifampin again (0.01 μg/ml) and one group without any antibiotic. The replicate cultures initially containing no antibiotic were incubated again without antibiotic. All cultures were then infected with *L. pneumophila* (10^4 CFU/ml) and CFU of *L. pneumophila* in the medium were determined daily for 4 d.

Assay for the effect of removal of antibiotics from monocyte cultures infected with L. pneumophila. Mononuclear cells (5×10^6) in 2 ml RPMI 1640 medium containing 15% fresh human serum were infected with *L. pneumophila* (5×10^3 CFU/ml) and incubated for 48 h at 37°C with erythromycin (1.25 μg/ml), rifampin (0.01 μg/ml), or without antibiotic. After 48 h, the leukocytes and bacteria were washed by centrifugation at 200 *g* for 10 min and 1,800 *g* for 15 min, and resuspended in RPMI 1640 medium containing 15% fresh human serum. The cultures were then incubated as follows. Cultures that initially contained antibiotic were split into two groups and incubated with or without the antibiotic as in the above assay. The cultures that initially did not contain antibiotic were incubated again without antibiotic. CFU of *L. pneumophila* in the medium were determined daily for 4 d.

Assay for determining how rapidly antibiotics inhibit the multiplication of L. pneumophila that are in the logarithmic phase of growth in monocytes. Mononuclear cells (5×10^6) were incubated in 35-mm plastic petri dishes (Falcon Labware) in 2 ml RPMI 1640 medium containing 5% fresh human serum for 1.5 h at 37°C in 5% CO_2-95% air to allow monocytes to adhere to the dishes. The monolayers were washed two times to remove the nonadherent lymphocyte-enriched fraction of the mononuclear cell population, and incubated in 2 ml RPMI 1640 medium containing 15% fresh human serum for 24 h. The monocyte monolayers were then infected with *L. pneumophila* and incubated for 24 h at 37°C in 5% CO_2-95% air until the bacteria were at the midlogarithmic phase of growth. The replicate monocyte monolayers were then vigorously washed three times with RPMI 1640 medium to remove extracellular bacteria. The cultures were incubated for 0, 1, 4, or 8 h in RPMI 1640 medium containing 10% fresh human serum and erythromycin (1.25 μg/ml), rifampin (0.01 μg/ml), or no antibiotic. At the end of the incubation, the monocytes were lysed by sonication and CFU of *L. pneumophila* were determined.

Electron microscopy. Mononuclear cells were infected with *L. pneumophila* and incubated for 48 h until the bacteria were in the logarithmic phase of growth in monocytes as in the "Assay for effect of antibiotics on *L. pneumophila* multiplication in monocytes" described above. The cultures were incubated with erythromycin (1.25 μg/ml), rifampin (0.01 μg/ml), or no antibiotic for 1, 4, or 24 h, and processed for electron microscopy (1).

Assay for determining if concentrations of antibiotics comparable to peak serum concentrations in humans are bactericidal for L. pneumophila located intracellularly in monocytes. Monocyte monolayers in 35-mm petri dishes were prepared as described in previous assays. The monolayers were infected with *L. pneumophila* and the bacteria allowed to multiply until they were in the logarithmic phase of growth. The monocytes were then vigorously washed to remove extracellular bacteria and 2 ml RPMI 1640 medium containing 10% fresh human serum added to each petri dish. Control monocytes were lysed by sonication to prevent further growth of *L. pneumophila* and incubated for 24 h without antibiotics. (During this period, CFU declined 0.30 logs in experiment 1 and 0.29 logs in experiment 2, Table II). The other monocytes were incubated for 24 h with 1.25 or 12.5 μg/ml erythromycin or 0.01–100 μg/ml rifampin and then lysed by sonication. The bacteria in all cultures were pelleted by centrifugation two times at 12,000 *g* (to wash away antibiotic from cultures treated with them), resuspended in 2 ml RPMI 1640 medium, and CFU of *L. pneumophila* per milliliter determined. Centrifugation resulted in a loss of 0.24 and 0.17 logs in experiments 1 and 2, respectively, Table II.

Assay for determining bactericidal effect of various concentrations of antibiotic on L. pneumophila multiplying extracellularly in broth. For comparison with the above assay, concentrations of antibiotic tested against *L. pneumophila* multiplying intracellularly in that assay were tested against *L. pneumophila* multiplying extracellularly in BYE broth. Midlogarithmic phase broth-grown *L. pneumophila* were prepared and added to plastic tubes containing BYE broth and antibiotic as in the "Assay for determining the MIC and MBC of erythromycin and rifampin for *L. pneumophila* multiplying extracellularly" except the final concentrations of antibiotic were 1.25 and 12.5 μg/ml erythromycin and 0.01, 0.02, 0.1, and 1.0 μg/ml rifampin. As a control for bacterial survival in a medium that does not support bacterial multiplication, *L. pneumophila* were added to plastic tubes containing EYB-BSA. Triplicate tubes were prepared for each concentration of bacteria and antibiotic. All tubes were sealed, rotated continuously at 37°C for 24

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h, and CFU per milliliter determined after serial dilution as in the "Assay for determining the MIC and MBC of erythromycin and rifampin for *L. pneumophila* multiplying extracellularly." In the experiment shown in Table III, CFU per milliliter of *L. pneumophila* in control tubes containing EYB-BSA decreased 0.4 log for inoculum A and 1.1 log for inoculum B.

Sonication of infected monocyte cultures. Monocytes were lysed by sonication with a 2.54-cm Diam high-gain disrupter horn attached to a sonicator (Heat Systems Ultrasonics, Inc., Plainview, NY). Sonication was performed under sterile conditions for 10 s continuously with the output control of the sonicator set at the 4 position. This amount of sonic energy lysed the leukocytes completely but did not reduce bacterial CFU (1).

RESULTS

MIC and MBC of erythromycin and rifampin multiplying extracellularly. We determined the MIC and MBC of erythromycin and rifampin for the *L. pneumophila* strain used in this paper (Philadelphia 1 strain) on bacteria multiplying extracellularly in BYE broth (Methods). The mean MIC for three experiments, each with two concentrations of bacteria, were 0.375 μg/ml erythromycin and 0.002 μg/ml rifampin (Table I). The mean MBC for two experiments, each with two concentrations of bacteria, were 1 μg/ml erythromycin and 0.009 μg/ml rifampin, higher than the MIC (Table I). CFU per milliliter were reduced ≥3 logs at the MBC, but cultures were not sterilized (Table I).

Erythromycin and rifampin inhibit the intracellular multiplication of L. pneumophila in human monocytes. We infected mononuclear cell cultures with *L. pneumophila* in the presence of 0–1.25 μg/ml erythromycin or 0–0.01 μg/ml rifampin and assayed the culture medium daily for CFU of *L. pneumophila* (Methods). *L. pneumophila* multiplied several logs in the presence of 0–0.125 μg/ml erythromycin but multiplication was completely inhibited by 1.25 μg/ml erythromycin (Fig. 1A). The MIC of erythromycin for *L. pneumophila* multiplying in broth was in this range (Table I).

L. pneumophila multiplied several logs in the presence of 0 or 0.001 μg/ml of rifampin, but multiplication was completely inhibited by 0.01 μg/ml rifampin (Fig. 2A). The MIC of rifampin for *L. pneumophila* multiplying in broth was also in this range (Table I).

Erythromycin (1.25 μg/ml) and rifampin (0.01 μg/ml) inhibited *L. pneumophila* multiplication whether added to mononuclear cells immediately before or

TABLE I
MIC and MBC of Erythromycin and Rifampin for L. pneumophila Multiplying Extracellularly

Experiment	Inoculum of *L. pneumophila*	Erythromycin			Rifampin		
		MIC	MBC	Δ log CFU/ml at MBC	MIC	MBC	Δ log CFU/ml at MBC
	CFU/ml						
1	5.8×10^5	0.25	—	—	0.004	—	—
	7.5×10^4	0.25	—	—	0.004	—	—
2	2.1×10^7	0.5	1	−3.9	0.002	0.016	−3.8
	3.5×10^5	0.25	1	−4.1	0.001	0.016	−5.5
3	2.5×10^7	0.5	1	−3.8	0.002	0.002	−4.4
	1.0×10^6	0.5	1	−3.4	0.0005	0.002	−3.8
	Mean	0.375	1		0.002	0.009	

In three independent experiments, *L. pneumophila* were grown to midlogarithmic phase in BYE broth and diluted in BYE broth to twice the concentrations indicated in the column entitled "Inoculum of *L. pneumophila*"; two concentrations of bacteria were studied in each experiment. The bacterial suspension was then diluted 50:50 with BYE broth containing twofold dilutions of antibiotic such that the final concentrations of antibiotic ranged from 0.0312 to 16 μg/ml erythromycin and from 0.000125 to 1 μg/ml rifampin. All measurements were performed in triplicate. The MIC was the lowest concentration of antibiotic inhibiting macroscopic growth and was determined as described in Methods; the values stated are for 48-h incubation in antibiotic-containing BYE broth. The MBC was determined in experiments 2 and 3. The MBC was the lowest concentration of antibiotic that resulted in ≥3 log mean decrease in CFU per milliliter for three replicate tubes after 48-h incubation in antibiotic-containing BYE broth. The mean log decrease in cultures containing the MBC level of antibiotic are stated in the columns entitled "Δ log CFU/ml at MBC."

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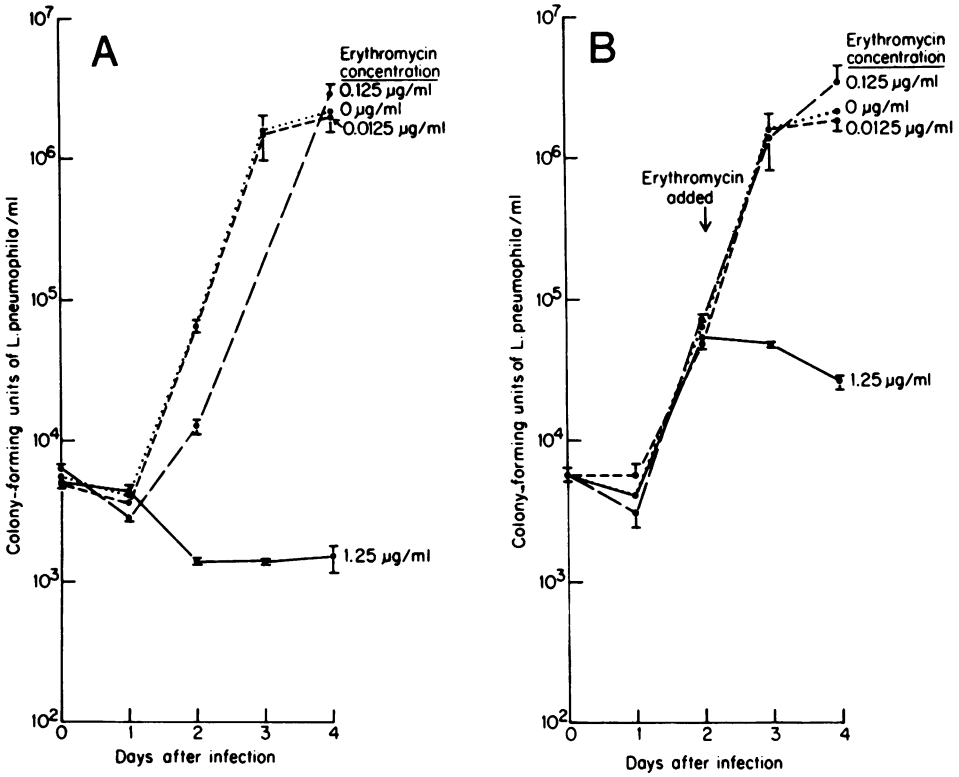

FIGURE 1 Effect of erythromycin on *L. pneumophila* multiplication in human monocytes. (A) Erythromycin inhibits the intracellular multiplication of *L. pneumophila*. *L. pneumophila* (5×10^3 CFU/ml) were incubated at 37°C in 5% CO_2–95% air with 5×10^6 mononuclear cells in 2 ml RPMI 1640 medium containing 15% fresh human serum and erythromycin in concentrations ranging from 0 to 1.25 μg/ml (Methods). CFU of *L. pneumophila* in the medium of each culture were determined daily. Each point represents the mean for two replicate cultures±SEM. (B) Erythromycin inhibits the intracellular multiplication of *L. pneumophila* that are in the logarithmic phase of growth in monocytes. Mononuclear cell cultures were prepared, infected with *L. pneumophila*, and assayed for CFU daily as in A except that erythromycin was not added until 48 h after the infection, when the bacteria were in the logarithmic phase of growth in monocytes. Each point represents the mean for two replicate cultures±SE.

after infection with *L. pneumophila* (data not presented).

Erythromycin and rifampin inhibit the intracellular multiplication of L. pneumophila in the logarithmic phase of growth in monocytes. We infected mononuclear cell cultures with *L. pneumophila* and allowed the bacteria to multiply until they were in the logarithmic phase of growth (Methods). We then added erythromycin (0–1.25 μg/ml) or rifampin (0–0.01 μg/ml) to the cultures. Erythromycin and rifampin inhibited multiplication of midlogarithmic phase *L. pneumophila* (Figs. 1B and 2B) and at the same concentrations that were inhibitory to lag phase bacteria (Figs. 1A and 2A).

Monocytes preincubated with antibiotics do not inhibit L. pneumophila multiplication after the antibiotics are removed. We preincubated mononuclear cells for 2 d without antibiotics or with inhibitory concentrations of erythromycin (1.25 μg/ml) or rifampin (0.01 μg/ml), washed the cultures to remove the antibiotics, and then infected the monocytes with *L. pneumophila* (Methods). *L. pneumophila* multiplied as readily in monocytes preincubated with inhibitory concentrations of erythromycin (Fig. 3A) or rifampin (Fig. 3B) as in monocytes preincubated without antibiotic.

Antibiotic inhibition of L. pneumophila multiplication in monocytes is reversible. To determine if *L. pneumophila* that have been inhibited from multiplying in monocytes by erythromycin and rifampin retain their capacity to multiply upon removal of the antibiotics, we infected mononuclear cell cultures and incubated the cultures with inhibitory concentrations of erythromycin (1.25 μg/ml) or rifampin (0.01 μg/ml) for 2 d (Methods). We then washed the cultures to remove antibiotic and continued the incubation with-

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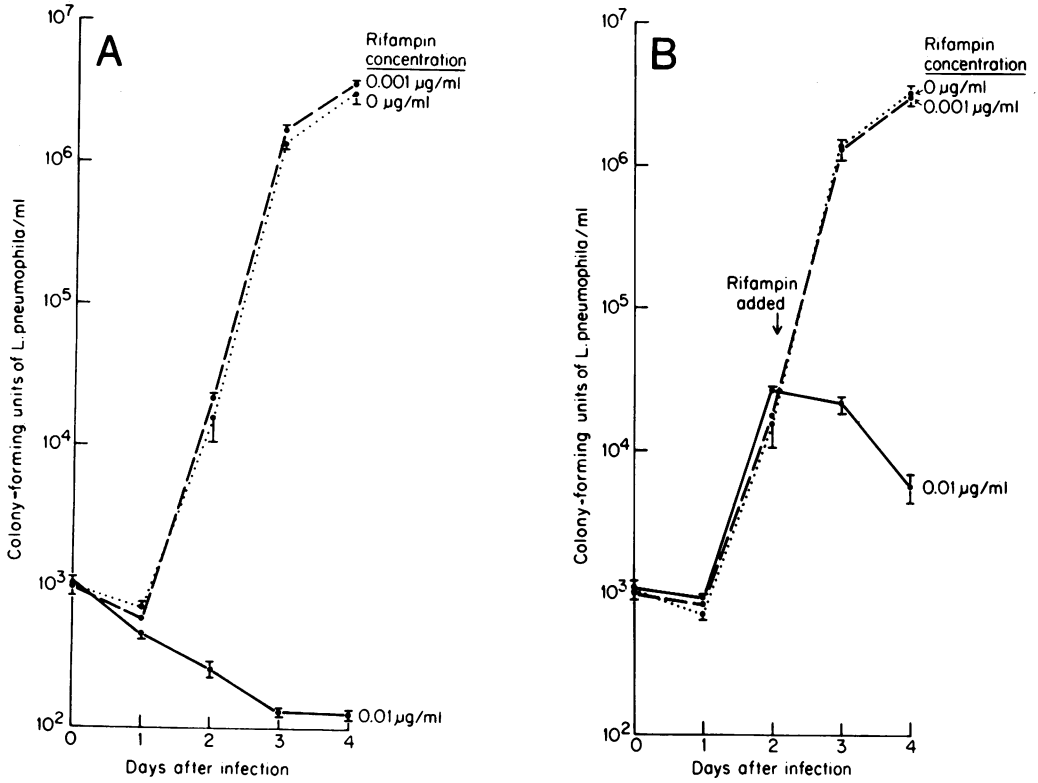

FIGURE 2 Effect of rifampin on *L. pneumophila* multiplication in human monocytes. (A) Rifampin inhibits the intracellular multiplication of *L. pneumophila*. Mononuclear cell cultures were infected with *L. pneumophila* (1×10^3 CFU/ml) and assayed for CFU daily as in Fig. 1A except that the cultures were incubated with rifampin in concentrations ranging from 0 to 0.01 μg/ml. Each point represents the mean for two replicate cultures±SEM. (B). Rifampin inhibits the intracellular multiplication of *L. pneumophila* that are in the logarithmic phase of growth in monocytes. Mononuclear cells were prepared, infected with *L. pneumophila*, and assayed for CFU daily as in A, except that rifampin was not added until 48 h after the infection, when the bacteria were in the logarithmic phase of growth in monocytes. Each point represents the mean for two replicate cultures±SE.

out antibiotic (Fig. 4). Control cultures were incubated either with or without antibiotic throughout the course of the experiment, i.e., before and after the cultures were washed.

In cultures incubated with inhibitory concentrations of erythromycin or rifampin, *L. pneumophila* multiplication was inhibited as in previous experiments (Fig. 4). When these antibiotics were removed from the cultures, *L. pneumophila* multiplied several logs (Fig. 4).

Thus, *L. pneumophila* are inhibited from multiplying by erythromycin and rifampin, but these bacteria retain their capacity to multiply when these antibiotics are removed.

Erythromycin and rifampin inhibit the intracellular multiplication of L. pneumophila in monocytes within 1 h of addition to midlogarithmic phase bacteria. To determine how rapidly erythromycin and rifampin inhibit *L. pneumophila* multiplication in monocytes, we infected monocytes in monolayer culture with *L. pneumophila*, allowed the bacteria to multiply until they were in the midlogarithmic phase of growth, washed the monolayers to remove extracellular bacteria, and added inhibitory concentrations of erythromycin (1.25 μg/ml) or rifampin (0.01 μg/ml) to the cultures. At 0, 1, 4, and 8 h thereafter, we lysed the monocytes by sonication, and assayed the cultures for CFU of *L. pneumophila* (Methods) (Fig. 5). *L. pneumophila* multiplication in monocytes was inhibited within 1 h of addition of erythromycin or rifampin (Fig. 5).

L. pneumophila multiplication in monocyte cultures during the first 8 h after infection probably represents multiplication of only bacteria that remain within the same monocyte in which they were located at 0 h and not in addition, multiplication of bacteria that have burst out of one monocyte and infected another. This is because (*a*) in an asynchronous culture, only a lim-

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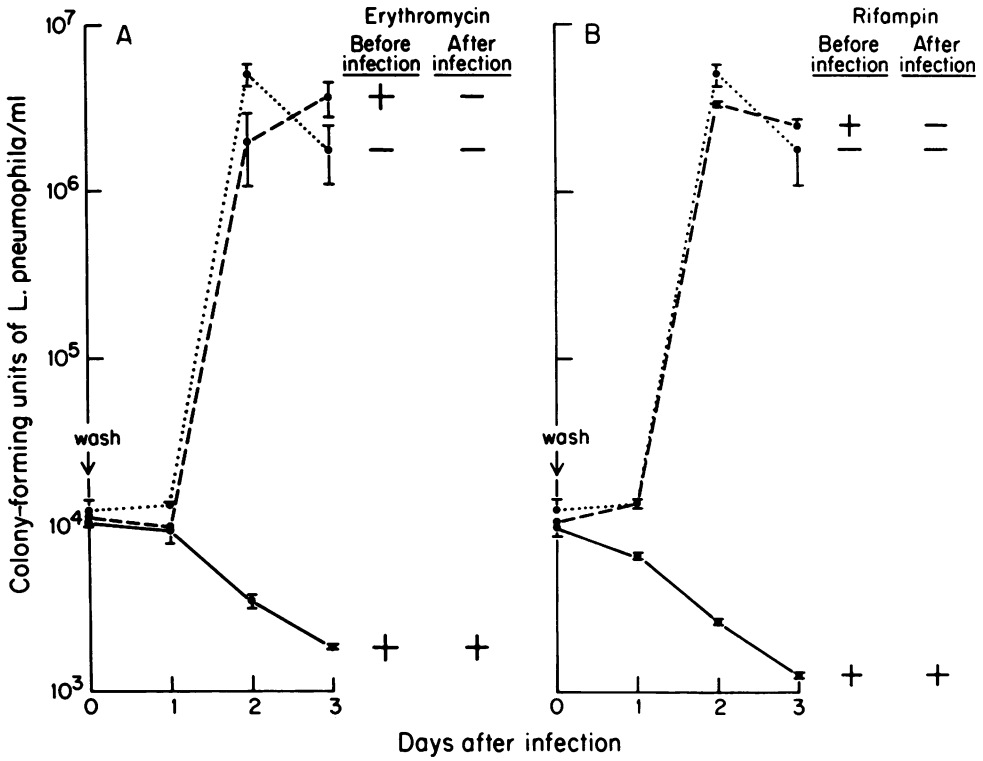

FIGURE 3 Monocytes preincubated with antibiotics do not inhibit *L. pneumophila* multiplication after the antibiotics are removed. Mononuclear cells (5×10^6) were incubated in plastic tubes in 2 ml RPMI 1640 medium containing 15% fresh human serum at 37°C in 5% CO_2–95% air for 48 h without antibiotic or with 1.25 μg/ml erythromycin (A) or 0.01 μg/ml rifampin (B). After 48 h, the cultures were washed to remove antibiotics (Methods). Cultures were then infected with *L. pneumophila* (10^4 CFU/ml) and incubated with or without antibiotics as follows. Cultures incubated without antibiotic before infection were incubated without antibiotic after infection (dotted lines). Cultures incubated with antibiotics before infection were split into two groups; one group was incubated after infection without antibiotic (dashed lines) and the other group was incubated after infection with the same antibiotic and at the same concentration as before infection (solid lines). (Following each line on the figure, "+" indicates antibiotic present and "−" indicates antibiotic absent before or after infection as indicated.) CFU of *L. pneumophila* in the medium were determined daily. Each point represents the mean for two replicate cultures±SE.

ited proportion of the monocytes are at the burst stage at any one time and only two doubling times occurred during the 8-h period of this experiment; thus, few monocytes were likely to have burst during this experiment; (*b*) infection of monocytes is very inefficient at low concentrations of extracellular bacteria (1), as was the case at time 0 h in this experiment, and even at high concentrations of extracellular bacteria, infection of monocytes is inefficient when cultures are held under stationary conditions and not shaken; thus, reinfection was unlikely to occur; and (*c*) data from other types of experiments indicate that *L. pneumophila* do not begin multiplying for at least 4 h, and usually for several hours longer, after entry into monocytes; thus, even if some reinfection did occur, few if any of these bacteria would have begun multiplying during the 8-h period of this experiment. For these reasons, the inhibition of *L. pneumophila* multiplication in antibiotic-treated cultures in this experiment probably represents inhibition, within 1 h, of bacteria located within the same vacuole in which they resided at time 0 h.

Since this experiment measures the effect of erythromycin and rifampin on intracellular bacteria only, the experiment demonstrates that, at the concentrations used, these antibiotics inhibit *L. pneumophila* multiplication but do not kill the bacteria to any great extent.

L. pneumophila inhibited from multiplying in monocytes by erythromycin or rifampin appear intact within membrane-bound cytoplasmic vacuoles. To examine the effects of inhibitory concentrations of

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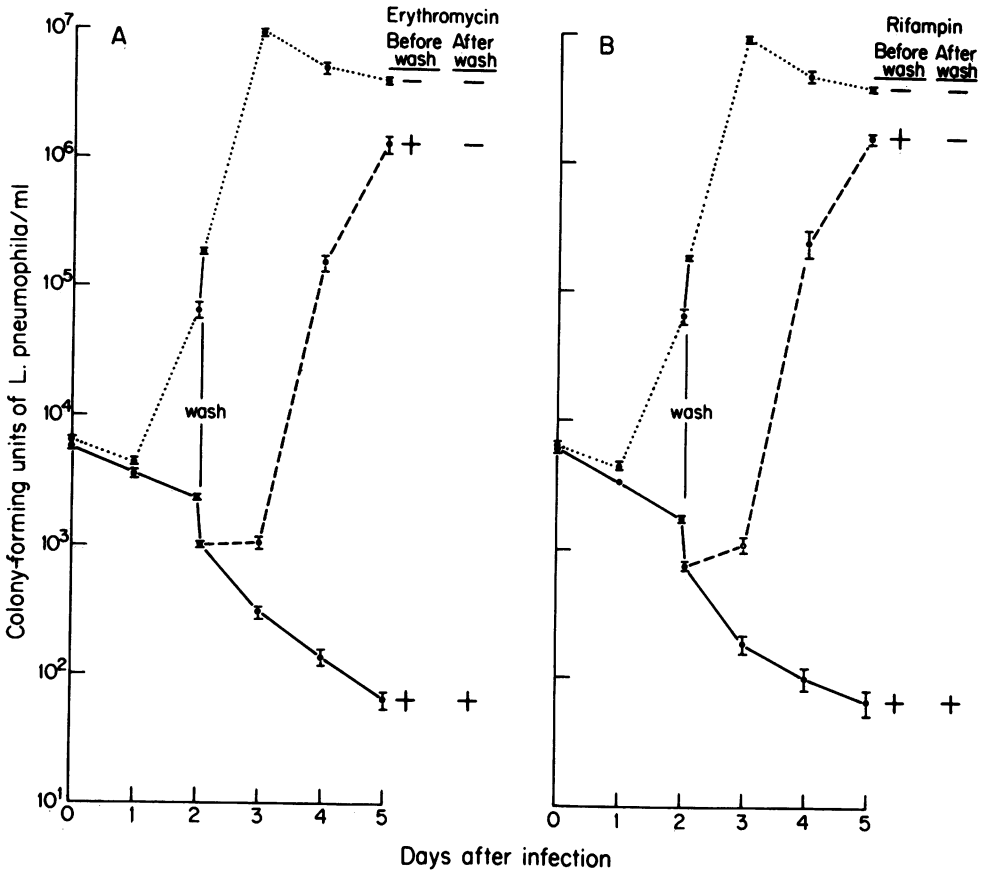

FIGURE 4 *L. pneumophila* inhibited from multiplying in monocytes by erythromycin and rifampin multiply after the antibiotics are removed. Mononuclear cells (5×10^6) in 2 ml RPMI 1640 medium containing 15% fresh human serum were added to plastic tubes, infected with *L. pneumophila*, and incubated for 48 h without antibiotic or with 1.25 μg/ml erythromycin (A) or 0.01 μg/ml rifampin (B). After 48 h the cultures were washed to remove antibiotics (Methods). Cultures incubated without antibiotic before washing were incubated without antibiotic after washing (dotted lines). Cultures incubated with antibiotics before washing were split into two groups after washing; one group was incubated without antibiotic (dashed lines) and the other group was incubated with the same antibiotic as before washing (solid lines). (Following each line on the figure, "+" indicates antibiotic present and "−" indicates antibiotic absent before or after washing as indicated.) CFU of *L. pneumophila* in the medium were determined daily. Each point represents the mean for three replicate cultures±SE.

antibiotics or midlogarithmic phase *L. pneumophila* in monocytes, we infected mononuclear cell cultures with *L. pneumophila*, allowed the bacteria to multiply to midlogarithmic phase, and then incubated the cultures for 1, 4, or 24 h with erythromycin (1.25 μg/ml), rifampin (0.01 μg/ml), or without antibiotic (Methods). We then examined the cultures by electron microscopy.

At 1, 4, or 24 h after the addition of antibiotics, *L. pneumophila* were found in membrane-bound cytoplasmic vacuoles within the monocytes. The bacteria appeared intact within the vacuoles and did not differ in appearance from bacteria in control monocytes not cultured in the presence of antibiotics. In monocytes treated for 4 h with erythromycin, rifampin, or no antibiotic, there were no significant differences in the appearance of vacuoles containing *L. pneumophila*; 93% of 15 such vacuoles in erythromycin-treated monocytes, 91% of 33 such vacuoles in rifampin-treated monocytes, and 88% of 25 such vacuoles in monocytes not treated with antibiotic were studded with ribosomelike structures; we have previously described this feature of *L. pneumophila*-infected monocytes (1).

L. pneumophila bacteria multiplying intracellularly in monocytes resist killing by peak serum concentrations of erythromycin or rifampin. Whereas erythromycin at 1.25 μg/ml and rifampin at 0.01 μg/

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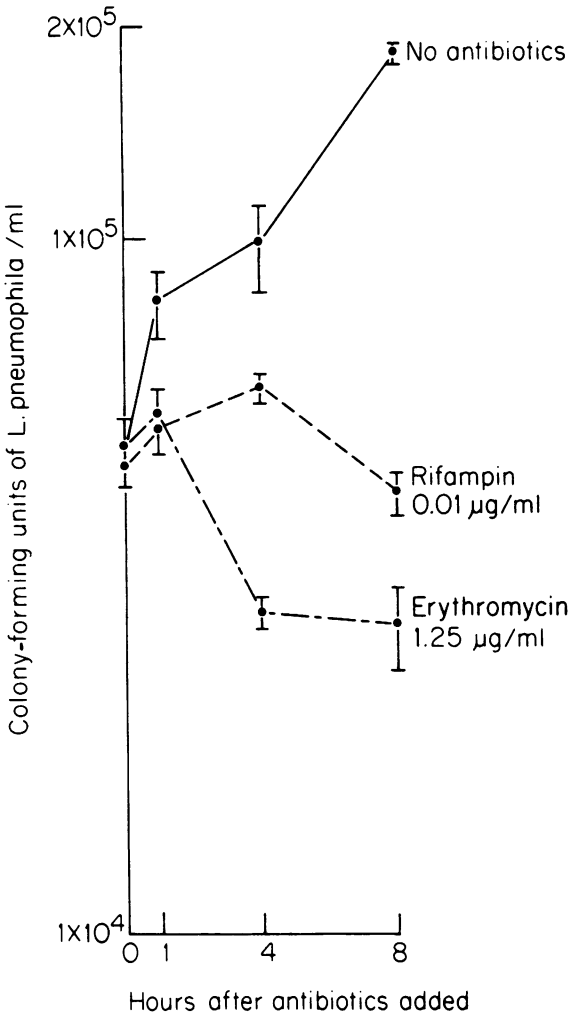

FIGURE 5 Erythromycin and rifampin inhibit the intracellular multiplication of *L. pneumophila* in monocytes within 1 h of addition to midlogarithmic phase bacteria. Monocyte monolayers in plastic petri dishes containing 2 ml RPMI 1640 medium and 15% fresh human serum were infected with *L. pneumophila*, incubated until the bacteria were in the midlogarithmic phase of growth, and vigorously washed to remove extracellular bacteria (Methods). The cultures were then incubated for 0, 1, 4, or 8 h with erythromycin (1.25 μg/ml), rifampin (0.01 μg/ml), or no antibiotic, as indicated. At the end of the incubation, the monocytes were lysed by sonication and CFU of *L. pneumophila* in the medium were determined. Each point represents the mean for three replicate cultures±SE.

ml were bacteriostatic but not bactericidal for *L. pneumophila* multiplying intracellularly in monocytes, these concentrations of antibiotic were bactericidal for *L. pneumophila* multiplying under cell-free conditions in broth. Therefore, we investigated whether higher concentrations of antibiotics, equal to or greater than peak serum concentrations in humans, are bactericidal for *L. pneumophila* located intracellularly in monocytes.

To examine this issue, we infected monocyte monolayers with *L. pneumophila* and allowed the bacteria to multiply to midlogarithmic phase. We then washed replicate cultures of monocytes to remove extracellular bacteria, and incubated the cultures for 24 h in the presence of 1.25 or 12.5 μg/ml of erythromycin or 0.01–100 μg/ml of rifampin. The peak serum concentration of intravenously administered erythromycin is ~10 μg/ml (17) and the peak serum concentration of orally administered rifampin is ~8 μg/ml (18). We omitted antibiotics from control cultures but lysed them by sonication to prevent further growth of *L. pneumophila* during the 24-h incubation. After 24 h, we lysed the monocyte cultures treated with antibiotics, washed the bacteria free of antibiotics by high-speed centrifugation, and assayed for CFU of *L. pneumophila* (Methods).

In erythromycin-treated cultures, CFU were slightly decreased (0.22–0.36 logs) in comparison with controls (Table II), but there was no significant difference in CFU between cultures treated with 1.25 μg/ml, 1.25 times the MBC for extracellularly multiplying *L. pneumophila* and cultures treated with 12.5 μg/ml, 10 times that concentration and a concentration comparable to peak serum levels.

In cultures treated with 0.01 μg/ml of rifampin, approximately the MBC for extracellularly multiplying *L. pneumophila*, CFU were only slightly decreased (0.05 or 0.24 logs) below the control level (Table II). In cultures treated with higher concentrations of rifampin, CFU were decreased in a dose-response fashion. However, even at a concentration of 100 μg/ml, 10 times the peak serum level, and 10,000 times the MBC for extracellularly multiplying *L. pneumophila*, CFU were reduced by only 1.3–1.7 logs. A control experiment showed that CFU of *L. pneumophila* were not decreased as a result of exposure of bacteria to high concentrations of rifampin after lysis of the monocytes or as a result of carry-over of rifampin with the bacteria through the centrifugation steps. Bacteria from cultures that had been sonicated and incubated for 24 h without antibiotics were divided into two groups and 100 μg/ml rifampin added to one of the two groups before the centrifugation steps. After centrifugation, there was no difference in the number of CFU of *L. pneumophila* in the two groups.

Thus, *L. pneumophila* resists killing by erythromycin and rifampin even when these antibiotics are at concentrations equal to or greater than peak serum concentrations in humans.

By way of comparison, we studied the effect of concentrations of antibiotics used in the studies presented in Table II on bacteria multiplying extracellularly

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TABLE II

Effect of Peak Serum Concentrations of Antibiotics on Midlogarithmic Phase L. pneumophilia Multiplying Intracellularly

Antibiotic	Concentration		CFU of *L. pneumophila*/ml after 24-h incubation with antibiotic preparation		Δ log‡	
	μg/ml	×MBC*	Expt. 1	Expt. 2	Expt. 1	Expt. 2
None	—	0	$1.4\pm0.3 \times 10^4$	$6.9\pm1.2 \times 10^3$	—	—
Erythromycin	1.25	1.25	$6.1\pm0.5 \times 10^3$	$4.1\pm0.8 \times 10^3$	−0.36	−0.22
	12.5	12.5	$6.4\pm0.8 \times 10^3$	$4.0\pm0.3 \times 10^3$	−0.35	−0.24
Rifampin	0.01	1	$1.3\pm0.2 \times 10^4$	$4.0\pm0.4 \times 10^3$	−0.05	−0.24
	0.02	2	$4.8\pm1.1 \times 10^3$	$1.9\pm0.2 \times 10^3$	−0.47	−0.56
	0.1	10	$2.1\pm0.2 \times 10^3$	$1.2\pm0.1 \times 10^3$	−0.82	−0.77
	1.0	100	$9.6\pm1.5 \times 10^2$	$5.9\pm0.4 \times 10^2$	−1.2	−1.1
	10	1,000	$3.3\pm0.3 \times 10^2$	$4.0\pm0.5 \times 10^2$	−1.6	−1.2
	100	10,000	$2.6\pm0.4 \times 10^2$	$3.4\pm0.2 \times 10^2$	−1.7	−1.3

Monocyte monolayers were prepared in 35-mm petri dishes, infected with *L. pneumophila*, and the bacteria allowed to multiply until they were in the logarithmic phase of growth (Methods). The monolayers were then washed to remove extracellular bacteria. Control monolayers were lysed by sonication to prevent further growth of *L. pneumophila* and incubated for 24 h without antibiotics. The other monolayers were incubated for 24 h with erythromycin (1.25 or 12.5 μg/ml) or rifampin (0.01–100 μg/ml) and then lysed by sonication. The bacteria in all cultures were washed by high-speed centrifugation to remove antibiotics from those cultures that had been treated with them, and CFU in each culture were determined as described in Methods. Each point represents the mean for three replicate monocyte cultures±SE. Expt., experiment.

* Mean MBC, as determined in Table I (1 μg/ml erythromycin and 0.009 μg/ml rifampin).

‡ (Log CFU in antibiotic-treated cultures) − (log CFU in control cultures not treated with antibiotics).

TABLE III

Effect of Antibiotics on Midlogarithmic Phase L. pneumophila Multiplying Extracellularly

Antibiotic	Concentration		CFU of *L. pneumophila*/ml after 24-h incubation with antibiotic preparation		Δ log	
	μg/ml	×MBC*	Inoculum A	Inoculum B	Inoculum A	Inoculum B
None	—	0	$1.0\pm.01 \times 10^7$	$8.6\pm0.9 \times 10^4$	—	—
Erythromycin	1.25	1	$1.4\pm0.8 \times 10^4$	$1.9\pm0.3 \times 10^3$	−2.9	−1.7
	12.5	12	$3.9\pm0.1 \times 10^3$	$2.2\pm0.4 \times 10^1$	−3.4	−3.6
Rifampin	0.01	1	$1.7\pm0.1 \times 10^2$	$6.7\pm2.7 \times 10^0$	−4.8	−4.1
	0.02	2	$2.0\pm0.6 \times 10^2$	$1.0\pm0 \times 10^1$	−4.7	−3.9
	0.1	10	$1.7\pm0.5 \times 10^2$	$3.3\pm2.7 \times 10^0$	−4.8	−4.4
	1.0	100	$9.3\pm4.0 \times 10^1$	0	−5.0	− >5.0

For comparison with Table II, concentrations of antibiotic tested against *L. pneumophila* multiplying intracellularly in Table II were tested against *L. pneumophila* multiplying extracellularly in BYE broth. Midlogarithmic phase broth-grown *L. pneumophila* were added to plastic tubes containing BYE broth and antibiotic such that the final concentrations of antibiotic were as indicated in the table and the final concentrations of bacteria were 2.5×10^7 CFU/ml (inoculum A) and 1.0×10^6 CFU/ml (inoculum B). As a control for bacterial survival in a medium that does not support bacterial multiplication, inocula A and B were suspended in EYB-BSA. The cultures were incubated 24 h and CFU per milliliter determined as described in Methods; data are the mean for three replicate tubes±SEM. In control tubes containing bacteria in EYB-BSA inoculum A decreased 0.4 log to $1.0\pm0.1 \times 10^7$ CFU/ml and inoculum B decreased 1.1 log to $8.6\pm0.9 \times 10^4$ CFU/ml during the 24-h incubation. The log difference between CFU per milliliter in these control cultures and CFU per milliliter in the antibiotic-treated cultures at the end of the 24-h incubation is given under the column entitled "Δ log".

* Mean MBC, as determined in Table I (1 μg/ml erythromycin and 0.009 μg/ml rifampin).

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rather than intracellularly. We incubated midlogarithmic phase bacteria for 24 h at 37°C in BYE broth containing the concentrations of antibiotics indicated in Table III. After 24 h, we determined the number of surviving bacteria and compared this with the number of surviving bacteria in a control culture incubated for 24 h without antibiotic in a medium that does not support *L. pneumophila* multiplication. In contrast to the situation where *L. pneumophila* were multiplying intracellularly (Table II), *L. pneumophila* multiplying extracellularly under cell-free conditions were highly susceptible to killing by erythromycin and rifampin (Table III); CFU per milliliter were reduced 1.7–3.6 logs below control levels in erythromycin-treated cultures, and 3.9–5 or more logs below control levels in rifampin-treated cultures.

DISCUSSION

We found that *L. pneumophila* multiplying intracellularly in human monocytes is inhibited from multiplying by concentrations of erythromycin and rifampin comparable to those that inhibit *L. pneumophila* multiplying extracellularly in artificial medium. However, *L. pneumophila* multiplying extracellularly is killed by relatively low concentrations of these antibiotics; the MBC are only three- to fivefold higher then the MIC. In contrast, *L. pneumophila* multiplying intracellularly is highly resistant to killing by erythromycin and rifampin even at concentrations comparable to or higher than peak blood levels in humans, levels 12 times the MBC for erythromycin and 10,000 times the MBC for rifampin.

Several differences between intracellular and extracellular bacteria may account for the disparity in their susceptibility to antibiotics. Bacteria multiplying extracellularly are directly exposed to antibiotics and under standardized physiologic conditions i.e., pH, osmolality, nutrient concentrations, et cetera, set by the investigator. Bacteria multiplying intracellularly are very differently situated; for an antibiotic to be effective against them, the antibiotic must first enter the host cell and gain access to the compartment in which they reside. Even then, whether or not the antibiotic can exert its characteristic antimicrobial effect is critically dependent upon the physiologic characteristics (e.g., the pH) of the pathogen's environment, which in turn are dependent upon other aspects of the host-parasite relationship such as whether the pathogen inhibits phagosome-lysosome fusion; *L. pneumophila* inhibits phagosome-lysosome fusion (19).

In addition to differences in location and environment, bacteria multiplying intracellularly may differ from bacteria multiplying extracellularly in their intrinsic susceptibility to antibiotics, since these bacteria differ from each other in other fundamental ways. For example, *L. pneumophila* growing extracellularly in artificial medium differ morphologically from bacteria growing intracellularly; in contrast to intracellularly growing bacteria, the extracellularly growing bacteria tend to form filaments and chains (20). Also, extracellularly grown bacteria have been found to be less virulent for guinea pigs and chicken embryos than intracellularly grown bacteria (21).

In vivo studies of antibiotic efficacy against *L. pneumophila* have also yielded results at variance with those obtained from studies involving *L. pneumophila* multiplying on artificial medium. Several antibiotics active against *L. pneumophila* growing on artificial medium have been found to lack efficacy against *L. pneumophila* in vivo (8–11). It is noteworthy that one such antibiotic, penicillin G, was reported by Johnson et al. (22) to be taken up very poorly by rabbit alveolar macrophages; in contrast, erythromycin and rifampin, which are active against *L. pneumophila* both in vivo and under cell-free conditions, were taken up readily by rabbit alveolar macrophages (8–11, 22). In fact, erythromycin and rifampin were among the few antibiotics tested that were concentrated in rabbit alveolar macrophages—erythromycin 20-fold and rifampin twofold (22). Thus, at least in the case of these three antibiotics to which *L. pneumophila* grown on artificial medium is sensitive, there is a good correlation between uptake by rabbit alveolar macrophages in vitro and efficacy in vivo.

Our earlier work indicates that in vivo, *L. pneumophila* multiplies only intracellularly (1). If so, then the present study indicates that in vivo *L. pneumophila* is resistant to killing by erythromycin and rifampin since both intracellularly multiplying bacteria and extracellular bacteria that are not multiplying are resistant to killing by these antibiotics. Since *L. pneumophila* probably multiplies only intracellularly in vivo, we suspect that the studies reported here of intracellularly multiplying *L. pneumophila* more likely reflect the in vivo situation than studies involving *L. pneumophila* multiplying extracellularly on artificial medium.

Our data show that *L. pneumophila* inhibited by erythromycin and rifampin retains its capacity to multiply in monocytes after the antibiotics are removed. This suggests that, in vivo, host defenses may be necessary to eliminate *L. pneumophila* bacteria from patients on antibiotics. Host defenses may also be required to inhibit multiplication of bacteria that survive a course of treatment. Previous work from this laboratory indicates that cell-mediated immunity plays a critical role in limiting the multiplication of *L. pneumophila* (5, 23). Viewed in this way, the role of antibiotics in Legionnaires' disease may be to suppress *L. pneumophila* multiplication long enough for the

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host to develop an effective immune defense against *L. pneumophila*.

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REFERENCES

1. 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.
2. Nash, T. W., D. M. Libby, and M. A. Horwitz. 1982. Legionnaires' disease bacteria multiply in human alveolar macrophages and activated alveolar macrophages inhibit multiplication. *In* Program of the 22nd Interscience Conference on Antimicrobial Agents and Chemotherapy, American Society for Microbiology, Miami Beach, FL, 4–6 October 81.
3. Horwitz, M. A., and S. C. Silverstein. 1980. Interaction of the Legionnaires' disease bacterium (*Legionella pneumophila*) with human phagocytes. I. *L. pneumophila* resists killing by polymorphonuclear leukocytes, antibody, and complement. *J. Exp. Med.* **153:** 386–397.
4. Horwitz, M. A., and S. C. Silverstein. 1980. Interaction of the Legionnaires' disease bacterium (*Legionella pneumophila*) with human phagocytes. II. Antibody promotes binding of *L. pneumophila* to monocytes but does not inhibit intracellular multiplication. *J. Exp. Med.* **153:** 398–406.
5. Horwitz, M. A., and S. C. Silverstein. 1981. Activated human monocytes inhibit the intracellular multiplication of Legionnaires' disease bacteria. *J. Exp. Med.* **154:** 1618–1635.
6. Fraser, D. W., T. R. Tsai, W. Orenstein, W. E. Parkin, H. J. Beecham, R. G. Sharrar, J. Harris, G. F. Mallison, S. M. Martin, J. E. McDade, C. C. Shepard, P. S. Brachman, and the Field Investigation Team. 1977. Legionnaires' disease. Description of an epidemic of pneumonia. *N. Engl. J. Med.* **297:** 1189–1197.
7. Meyer, R. P., and S. M. Finegold. 1980. Legionnaires' disease. *Annu. Rev. Med.* **31:** 219–32.
8. Fraser, D. W., C. Bopp, I. K. Wachsmuth, J. C. Feeley, and T. Tsai. 1978. Antibiotic treatment of guinea-pigs infected with agent of Legionnaires' disease. *Lancet.* **1:** 175–177.
9. Lewis, V. J., W. L. Thacker, C. C. Shepard, and J. E. McDade. 1978. In vivo susceptibility of the Legionnaires' disease bacterium to ten antimicrobial agents. *Antimicrob. Agents Chemother.* **13:** 419–422.
10. Thornsberry, C., C. N. Baker, and L. A. Kirven. 1978. In vitro activity of antimicrobial agents on Legionnaires' disease bacterium. *Antimicrob. Agents Chemother.* **13:** 78–80.
11. Thornsberry, C., and L. A. Kirven. 1978. β-Lactamase of the Legionnaires' bacterium. *Curr. Microbiol.* **1:** 51–54.
12. Aye, T., K. Wachsmuth, J. C. Feeley, R. J. Gibson, and S. R. Johnson. 1981. Plasmid profiles of Legionella species. *Curr. Microbiol.* **6:** 389–394.
13. Horwitz, M. A., and S. C. Silverstein. 1980. Influence of the *Escherichia coli* capsule on complement fixation and on phagocytosis and killing by human phagocytes. *J. Clin. Invest.* **65:** 82–94.
14. Wilkinson, H. W., D. D. Cruce, B. J. Fikes, L. P. Yealy, and C. E. Farshy. 1979. Indirect immunofluorescence test for Legionnaires' disease. *In* "Legionnaires": The Disease, the Bacterium, and Methodology. G. L. Jones and G. A. Hebert, editors. U. S. Department of Health and Human Services (DHHS), U. S. Public Health Service, Centers for Disease Control (CDC), Bureau of Laboratories, Atlanta, GA. Publication No. (CDC) 79-8375. 111.
15. Washington, J. A., II, and V. L. Sutter, 1980. Dilution susceptibility test: agar and macrobroth dilution procedures. *In* Manual of Clinical Microbiology. E. H. Lennette, A. Balows, W. J. Hauster, and J. P. Truant, editors. 3rd edition. American Society for Microbiology, Wash. DC. 453–458.
16. Anhalt, J. P., L. D. Sabath, and A. L. Barry. 1980. Special tests: bactericidal activity, activity of antimicrobics in combination, and detection of β-lactamase production. *In* Manual of Clinical Microbiology. E. H. Lennette, A. Balows, W. J. Hauster, and J. P. Truant, editors. 3rd edition. American Society for Microbiology, Wash., DC. 478–484.
17. Steigbigel, N. H. 1979. Erythromycin, lincomycin, and clindamycin. *In* Principles and Practice of Infectious Diseases. G. L. Mandell, R. G. Douglas, and J. E. Bennett, editors. John Wiley & Sons, Inc., New York. 290–300.
18. Kucers, A., and N. McK. Bennett. 1975. *In* The Use of Antibiotics. 2nd edition. William Heinemann Medical Books Ltd., London. 359.
19. Horwitz, M. A. 1982. Interaction between Legionnaires' disease bacteria and human monocytes: formation of a unique intracellular vacuole and inhibition of phagosome-lysosome fusion. *In* Program of the 22nd Interscience Conference on Antimicrobial Agents and Chemotherapy, American Society for Microbiology, Miami Beach, FL, 4–6 October 81.
20. Pine, L., J. R. George, M. W. Reeves, and W. K. Harrell. 1979. Physiology: characteristics of the Legionnaires' disease bacterium in semisynthetic and chemically defined liquid media. *In* "Legionnaires": The Disease, the Bacterium, and Methodology. G. L. Jones and G. A. Hebert, editors. U. S. Department of Health and Human Services (DHHS), U. S. Public Health Service, Centers for Disease Control (CDC), Bureau of Laboratories, Atlanta, GA. Publication No. (CDC) 79-8375. 28.
21. McDade, J. E., and C. C. Shepard. 1979. Virulent to avirulent conversion of Legionnaires' disease bacterium (*Legionella pneumophila*)—its effect on isolation techniques. *J. Infect. Dis.* **139:** 707–711.
22. Johnson, J. D., W. L. Hand, J. B. Francis, N. King-Thompson, and R. W. Corwin. 1980. Antibiotic uptake by alveolar macrophages. *J. Lab. Clin. Med.* **95:** 429–439.
23. Horwitz, M. A. 1982. Demonstration of cell-mediated immunity in Legionnaires' disease. *Clin. Res.* **30:** 369*a*.