Effect of levofloxacin, erythromycin or rifampicin pretreatment on growth of Legionella pneumophila in human monocytes


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Opsonophagocytic killing of some bacteria (Staphylococcus aureus, Pseudomonas aeruginosa) by phagocytes is enhanced by previous brief exposure of the organism to antibiotics. We studied the regrowth of Legionella pneumophila previously pretreated with levofloxacin, erythromycin and/or rifampicin in human monocytes. The MIC for the L. pneumophila isolate of levofloxacin, erythromycin and rifampicin was 0.03, 0.5 and 0.001 mg/L, respectively. Growth of L. pneumophila from buffered charcoal yeast extract (BCYE) agar for 24 h was subcultured into BYE broth containing from 0 to 4x MIC of levofloxacin, erythromycin or rifampicin. After incubation at 35°C in 5% CO₂ for 18 h, the organisms were washed and opsonized with 20% heat inactivated pooled normal human serum. Thereafter, L. pneumophila was exposed to human monocytes (5:1 ratio) previously adhered to wells in tissue culture plates containing RPMI and 10% fetal calf serum. After 0, 24, 48 and 72 h of incubation, quantitative cultures of lysed human monocytes were done on BCYE agar. Our results indicate effective inhibition on L. pneumophila at 0 h regardless of the antibiotic (levofloxacin, rifampicin or erythromycin) or their concentrations (1x, 2x or 4x MIC). At 24, 48 and 72 h, recovery and regrowth of L. pneumophila were both antibiotic- and concentration-dependent. In comparison with controls (no antibiotic pretreatment), peak regrowth of L. pneumophila pretreated with either 1x MIC of levofloxacin or erythromycin was delayed (48 versus 24 h) and reduced (30% of control peak regrowth). Regrowth of L. pneumophila pretreated with 1x MIC of rifampicin continued beyond 72 h. Pretreatment with levofloxacin at 4x MIC caused the greatest degree of growth inhibition (2 log₁₀). In contrast, at 72 h, regrowth of organisms pretreated with 4x MIC of erythromycin or rifampicin was less than peak control (P < 0.01) but greater than that seen with levofloxacin (P < 0.01). The rate and degree of regrowth of L. pneumophila pretreated with combinations of levofloxacin or erythromycin with rifampicin, or levofloxacin with erythromycin (all at 1x MIC) was similar to that seen with single drugs. Thus, significant delay and reduction of regrowth in this phagocytic system occurred with levofloxacin only. Prolonged exposure of the organism at 4x MIC levofloxacin concentrations was effective in suppressing regrowth of pretreated L. pneumophila in human monocytes.

Introduction

Enhanced opsonophagocytosis and killing of antibiotic-pretreated Gram-positive and Gram-negative microorganisms by phagocytic cells has been recognized. L. pneumophila is recognized as an intracellular pathogen which can multiply and survive in human macrophages. Thus, antibiotics with intracellular activity are needed for successful therapy. Comprehensive studies of the effects of extracellular exposure of L. pneumophila to antibiotics and subsequent survival after macrophage ingestion have not been described to date. It is known that macrolides and quinolones enter phagocytic cells. Levofloxacin is a new fluoroquinolone with enhanced antibacterial activity against L. pneumophila and excellent penetration into human monocytes.

The purpose of this study was to measure the effect of levofloxacin pretreatment of L. pneumophila with sub-

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inhibitory and supra-inhibitory concentrations of levofloxacin on the growth of surviving \textit{L. pneumophila} in human mononuclear cells. These observations are compared with the effects of erythromycin and rifampicin, used singly and in combination.

**Materials and methods**

\textit{Legionella pneumophila}

\textit{L. pneumophila} L-1033, serogroup I, isolated from the sputum of a patient with pneumonia was obtained from the Wadsworth Center for Research, New York State Department of Health, Albany, NY. The isolate was kept frozen in skim milk at -70°C until the experiment, then subcultured on buffered charcoal yeast extract (BCYE) agar, supplemented with 5% \( \alpha \)-ketoglutarate, and incubated at 35°C in 5% CO\(_2\). Colonies from the 48 h subculture were removed before each experiment, inoculated into BYE broth and incubated for 18 h at 35°C in a shaking water bath. Bacteria were then diluted to 10\(^{6}\) cfu/mL in BYE broth for incubation with antibiotics or kept in broth (control) until their addition to phagocytic cells.

\textit{Human mononuclear phagocytes (HMN)}

HMN were separated from heparinized venous blood obtained from normal volunteers using Histopaque-1077 (Sigma, Cincinnati, OH, USA). By trypan blue testing the viability of HMN was 95%. HMN were suspended in RPMI 1640 medium with 10% fetal calf serum (2 x 10\(^{6}\) HMN/mL). They were added to the wells of 24-well flat-bottom tissue culture plates (Corning, Belleville, NY, USA) and incubated for 2 h at 35°C in 5% CO\(_2\) to allow adhesion. Adhesion was >90% in each well before the addition of \textit{L. pneumophila}.

\textit{Opsonin}

Pooled heat inactivated normal human serum diluted to 20% (v/v) in RPMI 1640 was used to opsonize \textit{L. pneumophila} at 35°C for 1 h.

\textit{Antibiotics}

Levofloxacin was a gift of the Pharmaceutical Research Institute, Robert Wood Johnson Corp. (Princeton, NJ); erythromycin and rifampicin were obtained from Sigma. All antibiotic solutions were made fresh for each experiment according to the supplier’s instructions. Using the microdilution technique, the MIC (in mg/L) of each drug was determined in BYE broth.\(^5\) The MIC for \textit{L. pneumophila} L-1033 was 0.03, 0.5 and 0.001 mg/L of levofloxacin, erythromycin, and rifampicin, respectively.

Study design

\textit{L. pneumophila} L-1033 was grown for 18 h in BYE broth and added to 50 mL flasks containing 0, and 1/512 to 4 x MIC of levofloxacin, erythromycin and rifampicin as single drugs, and in the following combinations: levofloxacin + rifampicin, levofloxacin + erythromycin, and erythromycin + rifampicin at 1 x MIC. Flasks were incubated in a shaking water bath at 35°C for 20–180 min or for 18 h. The contents of the flasks were centrifuged at 10,000g for 10 min, washed in BYE broth, centrifuged, re-suspended in RPMI 1640 containing 20% pooled human serum, and opsonized for 60 min at 35°C in a bacterial density of 1 x 10\(^3\)/mL. After centrifugation, opsonized bacteria were washed and re-suspended in RPMI 1640 with 20% FCS at a final concentration of 2 x 10\(^5\) cfu/mL.

A antibiotic pretreated and control \textit{L. pneumophila} were added to adherent HMN at a 5:1 ratio of bacteria to cells, and incubated for 24, 48 and 72 h in duplicate wells. Before sampling, the supernates were removed by gentle washing of each well twice with RPMI 1640. The remaining HMN were lysed with distilled water and the contents of the well were plated on BCYE agar in duplicate. Plates were incubated for 48 or 72 h at 35°C and bacterial colonies were counted. The final number of surviving \textit{L. pneumophila} was determined for each duplicate well. Separate duplicate wells were sampled for each time point.

Statistical analysis and reporting of results

For test assays where \textit{L. pneumophila} were undetectable at day 0, but subsequently were detected in wells, we assigned the number 25 cfu/mL. The minimal sensitivity of our assay in detecting \textit{L. pneumophila} was 50 cfu/mL so that the true observed count, undetectable, was between 1 and 49 cfu/mL. Under control conditions (i.e. no antibiotic), during the 3 days of the experiment, counts of \textit{L. pneumophila} (cfu/mL) increased to a peak and then declined. In each assay, the peak log\(_{10}\) count was estimated by fitting a third-degree polynomial to the observed log\(_{10}\) counts using the method of least squares.\(^6\) The estimated maximum log\(_{10}\) count, based upon 13 assays, was 5.91 with a standard error of 0.12. On the average, the peak count occurred at day 0.9 (s.e. = 0.1). Statistical analysis utilized the analysis of variance technique\(^7\) and was done on the difference between the observed log\(_{10}\) (cfu/mL) and the estimated maximum log\(_{10}\) (cfu/mL) under control conditions. Results expressed, at each time point sampled (days 0, 1, 2, 3), the observed colony counts as a percentage of the estimated maximum control count. The level of significance was 0.05.

**Results**

Figure 1 demonstrates the effect of pretreatment of \textit{L. pneumophila} with 1 x, 2 x and 4 x MIC of levofloxacin and its capacity to regrow on days 1, 2 and 3. At day 0,
eight of 12 assays showed that the number of viable bacteria was below the detectable threshold of 50 cfu/mL. Beyond day 0, all three doses of levofloxacin caused concentration-dependent differential suppression of regrowth (P < 0.01). By day 3, regrowth of L. pneumophila was lowest at 4× MIC of levofloxacin compared with pretreated L. pneumophila with the control and 1× or 2× MIC (P < 0.05).

Figure 2 demonstrates the effect of pretreatment of L. pneumophila with 1×, 2× and 4× MIC of rifampicin and regrowth of the organism on days 1, 2 and 3. Eleven of 12 assays at day 0 demonstrated the marked effect of the drug by the absence of detectable viable bacteria (<50 cfu/mL). Regrowth of L. pneumophila pretreated with rifampicin at 1× MIC was higher than that of L. pneumophila pretreated with 2× or 4× MIC over the 3-day period (P < 0.01). After day 1, the greater rate of regrowth of the micro-organism pretreated with 4× MIC of rifampicin was observed in all assays. Peak regrowth time was not determined, as the assay was terminated at day 3.

Figure 3 demonstrates the effects of pretreatment of L. pneumophila with 1×, 2× and 4× MIC concentrations of erythromycin and regrowth of the organism on days 1, 2 and 3. All concentrations of erythromycin reduced the day 0 inoculum, but the effect of 2× and 4× MIC drug concentrations was greater than the effect of 1× MIC (P < 0.01). Pretreatment with 1× MIC reduced and delayed peak regrowth to 30% of the maximum control growth (P < 0.05).

Figure 4 compares the effects on regrowth of L. pneumophila after pretreatment with 4× MIC of levofloxacin, rifampicin or erythromycin. Twelve of 13 assays under these conditions demonstrated no detectable L. pneumophila at day 0, suggesting a marked degree of suppression
MIC of rifampicin for short time periods (20–180 min) had no 
1,14 It is known that fluoroquinolones are rapidly 
0.01). All 
MIC concentrations.
Changes 
MIC and 
1,9–16 
rifampicin, levofloxacin 
0.01). Sub-MIC concen-
terations of antibiotics for shorter time periods were ineffective. Furthermore, there was no evidence of additive or synergic effect when antibiotic combinations were studied at 1× MIC concentrations.

Several mechanisms that explain the effect of antibiotic pretreatment of L. pneumophila interaction with human monocytes are possible. They include post-antibiotic leucocyte enhancement effect (enhanced phagocytosis and/or enhanced intracellular killing of L. pneumophila), or prolonged growth inhibition by antibiotics (post-antibiotic effect) on intracellular L. pneumophila.9

Enhancement of phagocyte–bacterial interaction as an antimicrobial effect against other micro-organisms has been described and is known to occur even with sub-inhibitory concentrations of antimicrobials.1,3–6 Changes in bacterial morphology or external surface characteristics such as hydrophobicity or expression of opsonic binding sites have been suggested as mechanisms for increased phagocytosis of antibiotic-pretreated bacteria.1,4 In the present study, we did not perform direct measurements of phagocytosis of pretreated L. pneumophila. We studied the net regrowth of cell-associated bacteria. Although enhanced intracellular killing of pretreated bacteria is a possible mechanism for the delayed and decreased intracellular regrowth of L. pneumophila, it is known that human monocytes and macrophages in vitro and ex vivo lack the capacity to kill ingested L. pneumophila as opposed to other Gram-negative bacilli.

A likely explanation for the observations reported is a residual (post-antibiotic) effect of the antimicrobials used in the pretreatment of L. pneumophila. Only one previous report describes a slight effect of quinolone pretreatment with subinhibitory (0.25 MIC) concentrations on intracellular regrowth of L. pneumophila.15 In vitro and in the absence of monocytes, the fluoroquinolone antibiotics lomefloxacin and ciprofloxacin have demonstrated a post-antibiotic effect against L. pneumophila grown in BYE broth. The duration of this effect was 3.2 h for ciprofloxacin to 5.3 h for lomefloxacin, after a 1 h incubation at 4× MIC of the test strain.19

The post-antibiotic effect on L. pneumophila in phagocytes after incubation of ingested bacteria exposed to quinolones, macrolides and rifampicin has also been studied.19–22 It is known that fluoroquinolones are rapidly taken up and dissipated from both infected and non-infected phagocytes after the ambient antibiotic is removed.23 It is likely, therefore, that the suppression of regrowth observed intracellularly in our study was a result of the post-antibiotic effect on L. pneumophila. Minimal enhancement of intracellular killing of L. pneumophila by the phagocytes cannot be excluded.

**Discussion**

Our data demonstrate a decrease in both the rate and the degree of intracellular regrowth of L. pneumophila previously treated with clinically achievable concentrations of levofloxacin, rifampicin or erythromycin. The decrease and the delay in regrowth of L. pneumophila on day 3 was most evident after pretreatment with levofloxacin at 4× MIC for 18 h (P < 0.01). Sub-MIC concentrations of antibiotics for shorter time periods were ineffective. Furthermore, there was no evidence of additive or synergic effect when antibiotic combinations were studied at 1× MIC concentrations.

Figure 4. Geometric mean control of cfu/mL at days 0, 1, 2 and 3 after the pretreatment of L. pneumophila L-1033 with 4× MIC of levofloxacin (filled square), rifampicin (open square) and erythromycin (filled circle), and expressed as the percentage of the maximum number of cfu/mL under control conditions (no pretreatment).

of the inoculum before exposure to the mononuclear cells. However, at day 1 the percent average count was greater for erythromycin-pretreated organisms than for either levofloxacin- or rifampicin-pretreated L. pneumophila (P < 0.05). Organisms pretreated with 4× MIC of rifampicin demonstrated the most rapid rate of regrowth compared with erythromycin or levofloxacin. By day 3, L. pneumophila pretreated with levofloxacin had the least regrowth compared with erythromycin or rifampicin (P < 0.01). All three drugs decreased the counts of cfu/mL below the maximum estimated control peak by day 3, with levofloxacin at 1.7%, compared with 18% and 24.5% for rifampicin and erythromycin, respectively.

Combinations of levofloxacin + rifampicin, levofloxacin + erythromycin and erythromycin + rifampicin were tested by pretreating L. pneumophila at 1× MIC of each drug. A comparison of these combinations was made with the results of single drugs. There were no added or interfering effects of the drugs used in combination (data not shown).

Pretreatment of L. pneumophila with low concentrations of levofloxacin, rifampicin and erythromycin (1/512 to <1× MIC) for short time periods (20–180 min) had no effect on the regrowth of the organism (data not shown).
L. pneumophila regrowth in monocytes

The significance of our findings can be inferred from the understanding of the early events in the cellular pathogenesis of legionellosis. Although airborne macrophages and monocytes subsequently recruited from the circulation phagocyte L. pneumophila, this micro-organism is not killed effectively. Replication is followed by extracellular spread and invasion of other phagocytes, in which conditions for multiplication are excellent. Exposure of L. pneumophila to antimicrobials which act on both extracellular and as intracellular organisms during infection should, therefore, be beneficial. In the case of fluoroquinolones, including levofloxacin, a prolonged post-antibiotic effect could be anticipated on bacteria exposed before the ingestion by phagocytes, allowing a better possibility for phagocytic cells to achieve increased bactericidal capability. Rifampicin has been demonstrated to decrease the post-antibiotic effect of fluoroquinolones against Gram-negative bacilli and should be evaluated carefully in a similar test system against intracelullar and extra-cellular L. pneumophila. Less activity would be anticipated from erythromycin, with a post-antibiotic effect of <30 min.

In conclusion, pretreatment of L. pneumophila with levofloxacin at clinically achievable concentrations affects the rate and degree of regrowth of L. pneumophila ingested by human monocytes. A lesser effect is seen with rifampicin. Erythromycin is relatively ineffective at delaying the intracellular regrowth of pretreated L. pneumophila. Therefore, levofloxacin may have advantages in the treatment of legionellosis and should be evaluated in clinical studies.

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References


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