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Comparative Postantibacterial Activities of Pefloxacin, Ciprofloxacin, and Ofloxacin against Intracellular Multiplication of *Legionella pneumophila* Serogroup 1

PREMAVATHY RAJAGOPALAN-LEVASSEUR,[1]* ERIC DOURNON,[2] GUYLÈNE DAMERON,[1] JEAN-LOUIS VILDE,[1] AND JEAN-JACQUES POCIDALO[1]

Hôpital Claude Bernard, Université Paris VII, Institut National de la Santé et de la Recherche Médicale, Unité 13, Paris,[1] and Hôpital Raymond Poincaré, Garches,[2] France

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The inhibitory and postantibacterial activities of pefloxacin, ciprofloxacin, and ofloxacin against virulent *Legionella pneumophila* serogroup 1 were evaluated in cell-free and cellular models. In the absence of macrophages (with the tissue culture medium alone), bacterial numbers remained unchanged at 24 h in the presence of 0.1 μg of pefloxacin, ciprofloxacin, or ofloxacin per ml and 1.0 μg of pefloxacin per ml, whereas they were reduced in the presence of 1.0 μg of ciprofloxacin or ofloxacin per ml. Experiments to evaluate the postantibacterial effects of these drugs were therefore performed with concentrations of 0.1 μg/ml. In the cell-free model, brief exposure (1 h) of bacteria to each antimicrobial agent resulted in a transient decrease in numbers followed by logarithmic growth. In the cellular model, all three drugs (at 0.1 and 1.0 μg/ml) inhibited the intracellular multiplication of *L. pneumophila*. The intracellular postantibacterial effects of 0.1 μg of pefloxacin, ciprofloxacin, and ofloxacin per ml, which were left in contact with *L. pneumophila*-infected human macrophages for 24 h, were evaluated at various times after removal of the drugs. Pefloxacin was found to exhibit a significant inhibitory effect at 72 h, whereas following the removal of ciprofloxacin and ofloxacin, rapid bacterial multiplication occurred, leading to the destruction of the macrophage monolayer within 48 h. Thus, while pefloxacin, ciprofloxacin, and ofloxacin all inhibited the multiplication of *L. pneumophila* in human monocyte-derived macrophages, only pefloxacin exhibited a prolonged postantibacterial effect.

Three new fluoroquinolones, pefloxacin (6, 37), ciprofloxacin (17–19), and ofloxacin (35, 36), have been shown to be more active than erythromycin (6, 37) and josamycin (36) against *Legionella pneumophila* in cellular and experimental animal infection models. Other new difluorinated and trifluorinated quinolone agents have also been found to be active against *L. pneumophila* (7, 13, 16, 26, 31). We have previously demonstrated that pefloxacin shows a potent postantibacterial effect (PAE) against virulent *L. pneumophila* serogroup 1 in guinea pig infection and human monocyte-derived macrophage models (6, 37). Recently, Edelstein and Edelstein (12) have shown that contrary to ciprofloxacin, WIN 57273, a new quinolone antimicrobial agent, inhibits the growth of *L. pneumophila* grown in guinea pig alveolar macrophages in an irreversible manner.

The objective of this study was to evaluate and compare the intracellular PAEs of three quinolones, pefloxacin, ciprofloxacin, and ofloxacin, against *L. pneumophila*. The drug concentration that would inhibit but not kill the *Legionella* strain that was used, thus allowing the determination of intracellular PAE, was assessed first in a macrophage-free model. We then compared the intracellular inhibitory activities and PAEs of the three antimicrobial agents in a macrophage model.

MATERIALS AND METHODS

Bacteria. *L. pneumophila* serogroup 1 (strain Paris CB 81-13) was used in all experiments. This strain was isolated from the lung of a patient who died from Legionnaires disease during a nosocomial outbreak in Paris, France. Details of isolation and storage have been given elsewhere (33). Experimental cultures were performed at 35°C in an atmosphere containing 2.5% CO_2 and 95% humidity, unless otherwise stated, by using stock bacterial preparations obtained after two passages on buffered charcoal yeast extract agar supplemented with α-ketoglutarate (BCYE). Virulence was confirmed by intraperitoneal infection of guinea pigs and intracellular multiplication in human monocyte-derived macrophages as described previously (6, 33, 37).

Antimicrobial agents. The following commercially available injectable forms of antimicrobial agents were used: pefloxacin mesylate dihydrate (Laboratoire Roger Bellon, Neuilly-sur-Seine, France), ciprofloxacin chlorhydrate (Bayer Pharma, Puteaux, France), and ofloxacin sodium (Laboratoire Diamant, Paris, France). The stock solutions were diluted just before use. Preliminary experiments showed (i) no toxic effect against macrophages (as demonstrated by the trypan blue dye exclusion test) and (ii) bactericidal activity against intracellular *L. pneumophila* comparable to that of the powder forms supplied by the respective manufacturers.

Macrophage-free model. (i) MIC and MBC determinations. MICs and MBCs were assessed in yeast extract broth (YEB) (30, 34). In preliminary experiments the growth of *L. pneumophila* was quantified in YEB as follows. One portion of bacteria was thawed and diluted in sterile distilled water; 0.1 ml (containing about 10^5 bacteria) was added to 30 ml of YEB and cultured for 24 h. After 24 h of culture, the bacterial count (estimated by plating on BCYE agar) was 5×10^6/ml. In order to detect any interaction between the tested drugs and YEB (not conventionally used for MIC testing), the MICs for three reference strains (*Staphylococcus aureus* IP 76-25, *Escherichia coli* IP 76-24, and *Pseudomonas aerugi-*

* Corresponding author.

nosa IP 76-110) obtained from the Pasteur Institute (Paris, France) were determined in YEB and in Mueller-Hinton broth (MHB). Broth dilution MICs were ascertained by adding 1×10^7 *L. pneumophila* or control bacteria to 30 ml of medium (final bacterial concentration, 3.3×10^5/ml) in tissue culture flasks (Falcon; Becton Dickinson Labware, Oxnard, Calif.). The MIC was taken as the first drug concentration at which no visible growth occurred after 24 h (reference strains) or 48 h (*L. pneumophila*) of incubation at 35°C.

The MBC was determined only for *L. pneumophila*. The bacterial inoculum was prepared as described above for the MIC determinations. After 48 h of incubation with the drugs and 10-fold serial dilution in sterile distilled water, bacteria were counted by subculturing 0.1 ml of bacteria directly onto BCYE agar for 72 h at 35°C. Experiments were performed in duplicate. The MBC was taken as the lowest concentration which gave 99.9% killing.

(ii) Effect of removal of antimicrobial agents after 1 h on bacterial growth in YEB. Inocula were prepared in YEB prior to drug treatment and adjusted to a McFarland no. 0.5 standard. Approximately 10^7 *L. pneumophila* in 1 ml of YEB were incubated for 1 h with 0.1 μg of each antimicrobial agent per ml (two to four times the respective MICs). After 1 h, the cultures were centrifuged at 400 × *g* for 10 min. After carefully removing the supernatant, the pellet was washed twice with RPMI 1640 medium and suspended in 1 ml of YEB. Bacteria were counted in duplicate at 1, 24, 48, 72, 96, and 120 h following dilution in sterile distilled water. Cultures without antimicrobial agents were used to check that growth occurred in YEB. Further control cultures with antimicrobial agents were performed to ensure that growth of legionellae in YEB was inhibited by the drug concentrations that were used. Experiments were carried out in duplicate.

Macrophage model. (i) Monocyte-derived macrophages. Heparinized venous blood was obtained from healthy donors who were seronegative for anti-*L. pneumophila* antibodies. Mononuclear cells were separated by using a Ficoll-Hypaque gradient (Pharmacia Fine Chemicals AB, Uppsala, Sweden), and monocytes were isolated by adherence to a plastic substrate, as described previously (33). One milliliter of suspension containing approximately 10^6 monocytes per ml was layered onto sterile plastic tissue culture wells (Lab-Tek; Miles Laboratories, Inc., Elkhart, Ind.) containing 10% pooled normal serum and was left to adhere for 1.5 h. The adhering cells were washed gently with RPMI 1640 medium two more times to remove any remaining nonadherent cells. Fresh RPMI 1640 medium and 10% normal serum were added to the monolayers, which were cultured at 37°C in 5% CO_2 The medium was changed on alternate days, and a homogeneous layer of well-spread macrophages was obtained on day 7 of culture.

(ii) Immune serum. Immune serum was obtained from a patient who had recently recovered from Legionnaires disease and who was no longer receiving medication. The anti-*L. pneumophila* antibody titer, as measured by indirect immunofluorescence, was 1/512. Immediately after collection, the serum was filtered (pore size, 0.2 μm; Millipore Corp., Bedford, Mass.) and stored in portions at −70°C until use.

(iii) Normal serum. Serum samples from five healthy donors that were free of anti-*L. pneumophila* antibodies were pooled, filtered, and stored in portions at −70°C until use.

(iv) Infection of the macrophage monolayer. Seven-day-old monolayers (10^6 monocytes per well) were infected with *L. pneumophila* (10^7 CFU per well), and 10% fresh immune serum in RPMI 1640 medium (GIBCO, Paisley, Scotland) was added. The bacterium:cell ratio was 10:1. In previous studies we have found that, using this bacterium-to-cell ratio, 1 h after infection of monocyte-derived macrophages the mean number of intracellular legionellae is $1.55 \times 10^5 \pm 1 \times 10^5$ (mean ± standard deviation) (33, 37). After 1 h of culture, extracellular bacteria were removed by seven to eight washings with phosphate-buffered saline (with Ca^{2+} ions; pH 7.2), and intracellular bacteria were counted in duplicate wells after disruption of macrophages by incubation in 1 ml of sterile distilled water for 30 min with shaking. Noningested bacteria in other wells were washed out with seven to eight washings with phosphate-buffered saline. In an earlier study, we showed that after 1 h of incubation, bacteria were readily ingested and that after washing with phosphate-buffered saline, no cell-bound legionellae were demonstrable morphologically by direct fluorescence assay (38) or electron microscopy. Control bacterial cultures were run in the tissue culture medium alone to ensure that no multiplication occurred in the absence of macrophages. It has been shown previously (24, 37) that legionellae do not multiply under these conditions.

(v) Effect of antimicrobial agents on intracellular *L. pneumophila*. After removal of the extracellular bacteria, fresh medium containing 10% normal serum and with (0.1 and 1.0 μg/ml) or without antimicrobial agents was added to the monolayers to a final volume of 1 ml per well. The cultures were reincubated at 37°C with 5% CO_2 for 24 h, and extracellular and intracellular bacteria were counted. For each drug concentration, between two and eight experiments were done in duplicate. Control bacterial cultures with 0.1 and 1.0 μg of each drug per ml were performed to verify that no decrease in legionellae numbers occurred. Results were expressed in terms of an inhibition ratio: (total bacteria at 24 h with agent/total bacteria at 24 h without agent) × 100. Values lower than 10% were considered to indicate an inhibitory effect.

(vi) Withdrawal of antimicrobial agents. In some experiments, the medium containing 0.1 μg of drug per ml was withdrawn by seven to eight washings with phosphate-buffered saline after 24 h of contact with *L. pneumophila*-infected macrophages. Fresh drug-free nutrient medium (RPMI 1640 medium plus 10% normal serum) was added. Infected macrophages were further incubated, and intracellular and extracellular bacteria were counted at the times indicated above. Experiments were carried out in duplicate, with two to four experiments being performed at each time. Results were expressed by using an inhibition ratio: (total bacteria after 24 h of incubation with the drugs at various times after their removal/number of intracellular bacteria at 1 h) × 100. Values below 100% were considered to indicate an inhibitory effect.

(vii) Enumeration of CFU. Viable bacterial counts were determined after 1 and 24 h. After 24 h of incubation with the antimicrobial agents and at various times after their removal (every 24 h), the supernatant was aspirated and added to the intracellular bacteria that were obtained after hypotonic lysis with 1 ml of sterile distilled water. Tenfold serial dilutions of the suspensions were plated onto BCYE agar and incubated for 5 days before colony counting.

Statistical analysis. A one-way analysis of variance for unequal group sizes was applied to the data; a significance level (P) of <0.05 was chosen.

TABLE 1. MICs for *S. aureus*, *E. coli*, and *P. aeruginosa* strains in YEB and MHB

Antimicrobial agent	MIC (μg/ml)					
	S. aureus IP 76-25		*E. coli* IP 76-24		*P. aeruginosa* IP 76-110	
	YEB	MHB	YEB	MHB	YEB	MHB
Pefloxacin	0.25	0.25	0.125	0.125	0.50	0.50
Ciprofloxacin	0.12	0.12	0.016	0.002	0.10	0.25
Ofloxacin	0.12	0.25	0.05	0.05	0.50	0.50

RESULTS

Macrophage-free models. (i) MICs and MBCs of pefloxacin, ciprofloxacin, and ofloxacin for *L. pneumophila*. The MICs and MBCs of pefloxacin, ciprofloxacin, and ofloxacin for *L. pneumophila* Paris CB 81-13 in YEB after 48 h of incubation were 0.05, 0.025, and 0.05 μg/ml, respectively. The reference *S. aureus*, *P. aeruginosa*, and *E. coli* strains were inhibited by all three drugs in both YEB and MHB, indicating that the antimicrobial activity was not modified in this unconventional medium. The MICs for the reference strains are given in Table 1.

(ii) Effect of drug removal at 1 h on the multiplication of *L. pneumophila* in YEB. The number of legionellae ($\log_{10}$ CFU per milliliter) as a function of time in the absence and presence of antimicrobial agents is given in Fig. 1. In the absence of antimicrobial agents, a steady increase occurred until 18 h, followed by logarithmic growth from 24 h. In the presence of 0.1 μg of each drug per ml, the number of *L. pneumophila* decreased gradually with time compared with the controls. After brief exposure to the drugs, a transient decrease in bacterial numbers occurred, followed by logarithmic growth from 36 h.

Macrophage-associated models. (i) Control cultures. No change in *L. pneumophila* counts was observed after 24 h of incubation without macrophages or antimicrobial agents with RPMI 1640 medium plus 10% normal serum ($\log_{10}$ CFU for 12 experiments, 7.00 ± 0.2 at the outset and 7.13 ± 0.23 after 24 h). None of the drugs (concentration, 0.1 μg/ml) modified the bacterial count in the absence of macrophages (Table 2). At a concentration of 1.0 μg/ml, pefloxacin induced no change, whereas ciprofloxacin and ofloxacin led to a reduction in bacterial counts (Table 2). A concentration of 0.1 μg/ml was thus used to study the intracellular PAE.

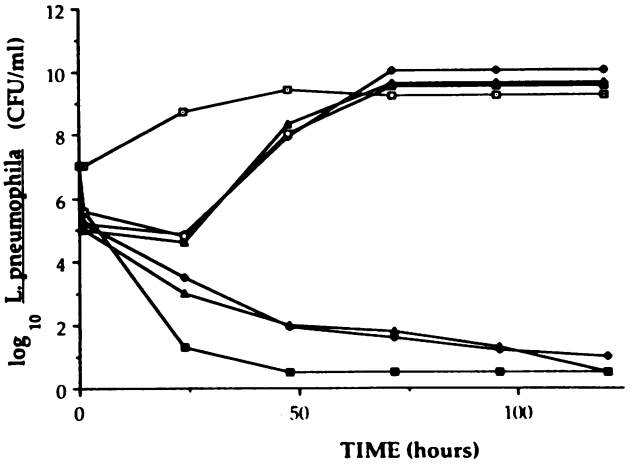

FIG. 1. Effect of drug removal at 1 h on the multiplication of *L. pneumophila* in YEB. Each point represents the mean of two values. ⊡, Control bacterial cultures without any drug. A concentration of 0.1 μg of pefloxacin (■), ciprofloxacin (◆), or ofloxacin (▲) per ml was present throughout the bacterial culture; a concentration of 0.1 μg of pefloxacin (□), ciprofloxacin (◇), or ofloxacin (△) was removed after 1 h.

(ii) Intracellular multiplication of *L. pneumophila* without antimicrobial agents. The mean ± standard deviation $\log_{10}$ intracellular CFU after 24 h of incubation (7.20 ± 0.5; 13 experiments) was approximately 180 times greater than the value at 1 h postinfection (5.10 ± 0.3; 11 experiments) ($P < 0.001$), i.e., a spontaneous intracellular multiplication at 24 h of between 1 and 2 $\log_{10}$ units. This variation that we observed could be due to the fact that monocytes from different donors were used.

(iii) Effect of drugs on intracellular multiplication of *L. pneumophila*. In the presence of 0.1 or 1.0 μg of each agent per ml, the number of bacteria at 24 h was reduced more than 10-fold (Table 2).

(iv) Effect of drug removal after 24 h of contact with *L. pneumophila*-infected macrophages. The total number of bac-

TABLE 2. Effects of pefloxacin, ciprofloxacin, and ofloxacin on intracellular multiplication of *L. pneumophila*[a]

Antimicrobial agent	Concn (μg/ml)	With macrophages[b]			Without macrophages		
		$\log_{10}$ CFU[c]	% Inhibition ratio[d]	No.[e]	$\log_{10}$ CFU[f]	% Inhibition ratio[d]	No.[e]
Pefloxacin	1	3.81 ± 0.22	1.10 ± 1.90	8	6.96 ± 0.23	73.6 ± 11.8	5
	0.1	3.90 ± 0.28	0.30 ± 0.30	3	7.18 ± 0.03	93.1 ± 28.2	2
Ciprofloxacin	1	3.90 ± 0.17	0.03 ± 0.02	3	5.96 ± 0.32	6.35 ± 2.30[g]	3
	0.1	4.75 ± 0.31	0.13 ± 0.09	3	7.06 ± 0.15	73.5 ± 13.40	3
Ofloxacin	1	4.04 ± 0.01	0.83 ± 0.50	2	5.90 ± 0.42	10.4 ± 9.50[g]	2
	0.1	4.46 ± 0.30	1.60 ± 2.40	6	6.94 ± 0.15	84.7 ± 48.8	5

[a] In the presence of macrophages, mean $\log_{10}$ CFU ± standard deviation of total bacteria at 24 h without antimicrobial agent was 7.20 ± 0.5 ($n = 13$).
[b] No significant difference between values for each concentration and each antimicrobial agent.
[c] Mean $\log_{10}$ CFU ± standard deviation of total bacteria at 24 h with antimicrobial agent.
[d] Mean percent inhibition ratio value ± standard deviation. Inhibition ratio was (total bacteria at 24 h with antimicrobial agent/total bacteria at 24 h without antimicrobial agent) × 100.
[e] Number of experiments.
[f] Mean $\log_{10}$ CFU ± standard deviation of total bacteria at 24 h with antimicrobial agent in the absence of macrophages.
[g] $P < 0.01$ versus a 1.0-μg/ml concentration of the respective antimicrobial agent.

TABLE 3. Effects of withdrawal of pefloxacin, ciprofloxacin, and ofloxacin at 24 h on intracellular multiplication of *L. pneumophila*[a]

Antimicrobial agent (concn, 0.1 μg/ml)	Mean % inhibition ratio ± SD (no.) at the following times (h)[b]				
	24	48	72	96	120
Pefloxacin	1.1 ± 0.7 (3.15 ± 0.27) (3)	12.6 ± 1.8 (4.18 ± 0.33) (4)[c,d]	79.8 ± 11.0 (5.09 ± 0.06) (4)[c]	747.0 ± 825.1 (5.82 ± 0.57) (4)[c]	4,677.4 ± 1,697.2 (6.84 ± 0.15) (4)[c]
Ciprofloxacin	43.0 ± 26.9 (4.75 ± 0.3) (3)	2,386.7 ± 2,744.2 (6.14 ± 0.82) (4)[c]	ND[e]	ND	ND
Ofloxacin	32.3 ± 23.0 (4.63 ± 0.33) (2)	2,939.8 ± 1,917.7 (6.57 ± 0.36) (3)[c]	ND	ND	ND

[a] The mean $\log_{10}$ CFU ± standard deviation for intracellular bacteria at 1 h was 5.10 ± 0.3 (n = 11), and for total bacteria at 24 h without antimicrobial agent it was 7.20 ± 0.5 (n = 13).

[b] Inhibition ratio: (total bacteria at each time after drug removal/intracellular bacteria at 1 h without drug) × 100. No. indicates number of experiments. Values in parentheses are mean $\log_{10}$ CFU ± standard deviation of total bacteria at each time after drug removal.

[c] $P < 0.001$ compared with the preceding column.

[d] $P < 0.001$ compared with ciprofloxacin and ofloxacin at the same time.

[e] ND, Not done, since macrophage monolayers were completely disrupted by this time.

teria after 24 h of contact with pefloxacin was inhibited significantly (10-fold) more than it was in the presence of ofloxacin or ciprofloxacin (Table 3). After removal of pefloxacin, intracellular bacterial numbers did not increase significantly during the first 72 h; thereafter, bacterial growth led to the compete destruction of the macrophage monolayers by 120 h. In contrast, removal of ciprofloxacin and ofloxacin led to rapid bacterial growth after 24 h and complete destruction of the macrophage monolayers by 48 h (Table 3).

DISCUSSION

L. pneumophila is a facultative intracellular pathogen which multiplies within phagocytic cells (24). The assessment of in vitro antibiotic activity is not always reliable for predicting antibiotic efficacy in the treatment of Legionnaires disease (14). This discrepancy may be linked to poor intracellular penetration, the activities of some antibiotics, or both (37). To overcome these problems, several investigators have developed animal (6, 11, 18, 19, 22, 26, 29, 35, 36) and cell (17, 23, 25, 27, 36, 37, 40) models of *Legionella* infections. We have previously used the macrophage model to assess the activity of antibiotics against *L. pneumophila* that was multiplying intracellularly (37).

In this study, the activities of three quinolone agents, pefloxacin, ciprofloxacin, and ofloxacin, against *L. pneumophila* were assessed in cell-free and macrophage models. The MICs and MBCs of the three drugs were very similar. However, in cell-free experiments with medium that was not supportive of bacterial growth, 1.0 μg of pefloxacin per ml was ineffective against legionellae, whereas ciprofloxacin and ofloxacin effectively reduced bacterial numbers (Table 2). In contrast, the three drugs were equally effective in inhibiting the intracellular multiplication of legionellae (Table 2).

In the macrophage model, while intense bacterial multiplication at 24 h after the removal of ciprofloxacin and ofloxacin led to the destruction of the macrophage monolayers by 48 h, bacterial regrowth only occurred 72 h after the removal of pefloxacin, reflecting a probable intracellular PAE of pefloxacin. This prolonged PAE was observed only in the presence of macrophages since, in the cell-free model, the removal of the drug was followed by logarithmic growth in medium that was supportive of bacterial growth after a short lag period. According to the definition of PAE (2), the time lag observed for regrowth of bacteria after drug removal (compared with that of bacteria in drug-free cultures) suggested a PAE of 18 h in cell-free YEB medium (Fig. 1). Although all three drugs showed a short PAE in vitro, only pefloxacin showed a prolonged intracellular PAE. These results indicate that cellular models may be more reliable for predicting the efficacies of antibiotics in the clinical setting.

Several investigators have shown that quinolone antimicrobial agents are more active than erythromycin against *L. pneumophila* in cellular and experimental infection models (6, 12, 18, 37). In earlier studies, we have shown that pefloxacin is more active than erythromycin against intracellular *L. pneumophila* (37) and in experimental *Legionella* infections of guinea pigs (6). In both these models, pefloxacin exhibited a persistent inhibitory activity. Similar inhibition of bacterial regrowth in guinea pig alveolar macrophages by WIN 57273, a new quinolone antimicrobial agent, has been observed (12). Erythromycin does not exhibit such a PAE in a macrophage model (12, 37), within monocytes (25), or in experimental infections of guinea pigs (6). The intracellular PAE may be responsible for the in vivo effects observed in guinea pigs and for the higher activities of quinolone antimicrobial agents compared with that of erythromycin.

The PAE of pefloxacin against intracellular *L. pneumophila* may be explained by the persistence of the drug within the cells or by a particularly potent effect on intracellular bacteria, which was reflected by the lag period of 3 days following drug removal. With regard to the persistence of the drug, it has been shown that the uptake and release of quinolone antimicrobial agents by monocyte-derived macrophages are both very rapid (8–10; M. B. Carlier, B. Scorneaux, A. Zenebergh, and P. M. Tulkens, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 622, 1987), thereby ruling out the possibility that the persistent inhibitory activity against intracellular *L. pneumophila* is due to residual pefloxacin. Moreover, in experimental infections of guinea pigs, pefloxacin exhibits a persistent inhibitory effect, despite negligible amounts of drug in blood and lungs at 17 h posttreatment (6). Conversely, erythromycin (which is known to accumulate within cells [21, 28, 32]) does not prevent bacterial regrowth in either cell or animal models (6, 12, 25, 37). It can be argued that the PAE of pefloxacin might simply represent increased

intracellular potency since, at 24 h after drug removal, the number of bacteria remaining in the macrophages was 10-fold less than those in experiments with ciprofloxacin or ofloxacin (Table 3).

In addition to PAE, other factors which should be taken into account with regard to intracellular drug activity include (i) modulation of the bactericidal mechanisms of phagocytes (1, 3–5, 15, 20, 27), (ii) changes in intracellular pH, (iii) fixation and irreversible damage of bacteria (39), and (iv) distribution and persistence of the drugs within phagosomes, where legionellae multiply.

The results of this study suggest that conventional MIC, MBC, and in vitro PAE data should be interpreted with caution, at least regarding intracellular pathogens. The significance of the prolonged inhibition of regrowth of legionellae after the removal of pefloxacin is not clear. It has been suggested that this property could be of use in antimicrobial dosing regimens (2). However, the clinical importance of the quinolone PAE remains to be established and will require further studies in animals and humans.

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