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Cellular uptake, localization and activity of fluoroquinolones in uninfected and infected macrophages

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Pefloxacin, like other fluoroquinolones, accumulates in macrophages and several other types of nucleated cells (but not in erythrocytes). Upon fractionation of macrophage homogenates by isopycnic centrifugation in sucrose gradients, fluoroquinolones are not found associated with any specific cellular structure. We have compared the activities of pefloxacin and roxithromycin against intracellular *Staphylococcus aureus* in mouse J774 macrophages. Pefloxacin was significantly more active for equivalent intracellular drug concentrations (i.e. expressed by reference to the respective MICs of the drugs as determined in broth), suggesting differences in intracellular availability and/or capacity of the drugs to express their activity in the intracellular environment. The difference was enhanced by incubating the cells in acidic medium. We have also examined the cellular pharmacokinetics and intracellular distribution of pefloxacin in uninfected and *Legionella pneumophila* infected guinea pig macrophages. In contrast to uninfected cells from which pefloxacin was quickly released, macrophages infected with legionella retained approximately 20–30% of the accumulated pefloxacin after a 60-min wash-out. Cell fractionation studies indicated that the drug remaining in cells was associated with components of high buoyant density. These fractions also contained [^{3}H] if cells had been incubated with [^{3}H] labelled legionella (by in-vitro exposure to [^{3}H]-thymidine, before phagocytosis). These results suggest that part of the intracellular pefloxacin becomes associated with legionella, or with legionella-containing cytoplasmic structures.

Introduction

Penetration and accumulation of an antimicrobial within phagocytic cells are important determinants, among others, of its activity against intracellular bacteria. After having penetrated into cells, however, an antibiotic must also be able to reach the bacteria which are often, but not always, localized in specialized vacuoles. The drug must then remain stable and capable of expressing its activity in the physico-chemical conditions prevailing at this specific site. Finally, the bacteria must be in a metabolic state that

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renders them sensitive to the drug under study. Inability to meet one or several of these conditions will result in the drug being largely inactive. For example, β-lactam antibiotics penetrate phagocytes but fail to accumulate to protective concentrations in most cases (Holmes *et al.*, 1966; Prokesh & Hand, 1982; Renard *et al.*, 1987). β-Lactams are also poorly active against slowly or non-dividing bacteria. Aminoglycoside antibiotics accumulate only very slowly in phagocytes, and are distributed almost exclusively in lysosomes where the acidic pH largely abolishes their activity (Tulkens & Trouet, 1978). Lincosamines such as clindamycin (but not lincomycin) show great cellular accumulation (Hand & King-Thompson, 1982; Prokesh & Hand, 1982; Hand *et al.*, 1984), but, surprisingly, display little or no activity against sensitive bacteria (Hand & King-Thompson, 1986; Sanchez, Ford & Yancey, 1986; Scorneaux, Zenebergh & Tulkens, 1989) for reasons so far unknown. Macrolides and fluoroquinolones are accumulated by phagocytes (Easmon & Crane, 1985; Martin, Johnson & Miller, 1985; Carlier, Zenebergh & Tulkens, 1987*a*; Carlier *et al.*, 1987*b*; Pascual, Garcia & Perea, 1989), and are reported to be active against several sensitive intracellular bacteria (Fitzgeorge *et al.*, 1985; Easmon, Crane & Blowers, 1986; Vildé, Dournon & Rajagopalan, 1986; Carlier *et al.*, 1989; Scorneaux *et al.*, 1989). In the present study, we have evaluated the accumulation and subcellular localization of pefloxacin in cells in culture and its release from these, and have correlated these parameters with its activity against two types of intracellular bacteria, namely *Staphylococcus aureus* and *Legionella pneumophila*. Comparisons with other fluoroquinolones and with a macrolide (roxithromycin) have also been made in some instances. The purpose of the work was to develop approaches to comparison and assessment of antimicrobials used against intracellular infections.

Methods

Cells

J774 macrophages, unelicited guinea pig peritoneal macrophages, rat embryo fibroblasts, and human polymorphonuclear leucocytes were obtained and cultivated, or maintained, by techniques similar to those described by us earlier (Tulkens, Beaufay & Trouet, 1974; Canonico *et al.*, 1978; Carlier *et al.*, 1987*a*). Human red blood cells were obtained from normal volunteers.

Accumulation studies

Accumulation and release studies of fluoroquinolones and of roxithromycin were performed using [^{14}C]-labelled compounds as described by Carlier *et al.*, (1987*a*) and Renard *et al.* (1987). We measured the protein and radioactivity content of cell pellets and we calculated the apparent cellular to extracellular concentration ratios of the drugs assuming that 1 mg of cell protein corresponded to a cell volume of 5 μl for nucleated cells, a value close to that experimentally determined for mouse macrophages (Steinman, Brodie & Cohn, 1976) and rat embryo fibroblasts (Tulkens & Trouet, 1978), and to a volume of 3·5 μl for erythrocytes. This calculation also assumes that cell-associated drugs are homogeneously distributed within the cell volume, which may not be correct, and we therefore refer to their cellular/extracellular concentration ratio as being an apparent one.

Fractionation of J774 macrophages

Subcellular distribution studies of fluoroquinolones in uninfected J774 macrophages were performed on post-nuclear supernatants prepared from homogenates of cells by the techniques described by Canonico *et al.* (1986), Aubert-Tulkens, Van Hoof & Tulkens (1979) and Renard *et al.* (1987) with minor adaptations. The mode of representation is that recommended by Leighton *et al.* (1968) and Beaufay & Amar-Costesec (1976), in which the abscissa is the density scale of the gradient, and the ordinate shows the frequency of each constituent assayed. To compare different drugs (Figure 2), we used the relative frequency so that the total area of each histogram is equal to 1. To compare the same constituent assayed in different experiments, and for which the amount associated with cells varied (Figure 4), we used the *absolute* frequency (Bulychev, Trouet & Tulkens, 1978), so that the total surface area of the corresponding histograms is proportional to the amount of this constituent collected in the fractions.

Infection of J774 macrophages by S. aureus *and influence of antibiotics on cell-associated* S. aureus *growth*

J774 macrophages were infected with *S. aureus* (a clinical isolate of bovine origin kindly donated by Professor De Vries, Rijksuniversiteit Ghent, Ghent, Belgium) using a technique derived from that described by Sanchez *et al.* (1986) for bovine polymorphonuclear leucocytes. Bacteria were exposed to normal fresh human serum for opsonization before phagocytosis. After phagocytosis, macrophages were washed, exposed to lysostaphin (4 units/ml) for 15 min at 37°C and reincubated for up to 24 h in medium without antibiotic, or with increasing amounts of antibiotic. At regular intervals, samples of cells were washed, re-exposed to lysostaphin for 15 min at 37°C, washed, scraped from the culture plate and pelleted by low-speed centrifugation. Cell pellets were assayed for protein and for colony forming units by plating on nutrient agar. It was established in preliminary experiments that addition of oxacillin or gentamicin (at extracellular concentrations of 10 × MIC) during the incubation time did not reduce by more than 10% the growth of cell-associated *S. aureus* under these circumstances. The influence of pH on the activity of antibiotics on growth of cell-associated *S. aureus* was examined by running the experiments in media the pH of which was maintained constant throughout by replacing the CO_2/bicarbonate buffer by 10 mM Na^+/K^+ phosphate buffer, and by appropriate addition of small amounts of diluted NaOH at regular intervals.

Infection of macrophages by L. pneumophila *and fractionation of legionella-infected cells*

Infection of guinea pig macrophages by *L. pneumophila* (strain Paris CB/81–13; serogroup 1, kindly donated by Professor Vildé, Paris, France) was performed according to Fitzgeorge *et al.* (1985) and Vildé *et al.* (1986) using human serum from patients recovering from Legionnaire's disease for opsonization. *L. pneumophila* cells were labelled by cultivating them for 24 h at 37°C on BCYE Agar in the presence of [^{3}H]–labelled thymidine (2 mCi/dish) followed by collection of the bacteria and by a 5 h incubation of the bacterial suspension in thymidine-free TC 199 medium supplemented with 10% human serum at 37°C. The final preparation showed a radioactivity of

approximately 6000 cpm/10^6 cfu, which was precipitable to more than 95% in 5% trichloroacetic acid. For fractionation studies, cells were exposed to opsonized legionella for 1 h at 37°C, washed twice for 30 min in bacteria-free medium at 37°C, and further incubated in the presence of pefloxacin (5 mg/l) for up to 6 h. Cells were then washed, reincubated in drug-free medium for 1 h and collected for fractionation. We used a protocol similar to that developed for J774 macrophages (see above), but homogenization of cells was performed by the 'needle/syringe' approach developed by Canonico *et al.* (1978). A detailed description of the procedure and data demonstrating the essential intactness of the subcellular organelles in homogenates of legionella-infected macrophages will be given in a forthcoming publication.

Antibiotics, labelled and unlabelled, were kindly donated by or supplied under the control of their corresponding manufacturers (pefloxacin, Rhône-Poulenc, Paris, France; lomefloxacin, Searle Inc., Chicago, Ill.; ciprofloxacin, Bayer AG, Wuppertal, Federal Republic of Germany; ofloxacin, Hoechst AG, Darmstadt, Federal Republic of Germany; fleroxacin, Hoffman-La Roche, Basle, Switzerland; roxithromycin, Roussel-Uclaf, Paris, France). We checked by thin-layer chromatography techniques that the radioactivity was, and remained, associated with the corresponding drug under the conditions of our experiments, both in the culture media and in the cells. Other materials were of the same origin and purity as reported in our previous publications (Carlier *et al.*, 1987*a*; Renard *et al.*, 1987).

Results

Accumulation and distribution of fluoroquinolones in J774 macrophages

Figure 1 shows that J774 macrophages accumulated pefloxacin, lomefloxacin or fleroxacin to 5 to 9 times their extracellular concentration, when incubated at 37°C in

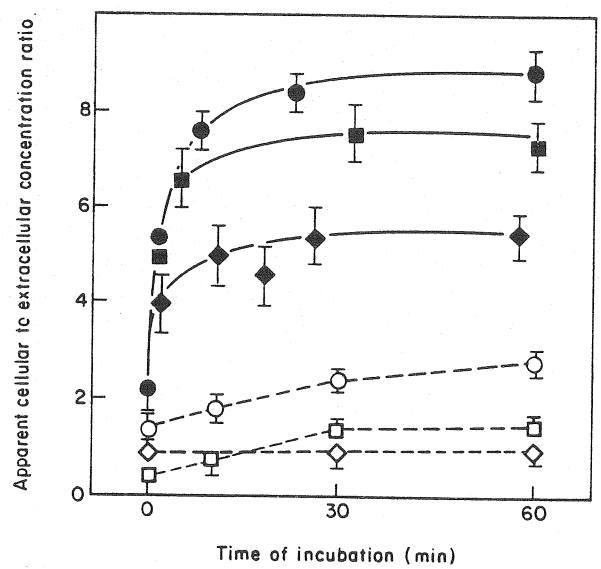

Figure 1. Apparent cellular to extracellular concentration ratios of three fluoroquinolones in J774 macrophages upon incubation at 37°C (closed symbols) or 4°C (open symbols), at an extracellular concentration of 10 mg/l. Values are given ± S.D. ($n = 3$). ●, ○, Pefloxacin; ■, □, lomefloxacin; ◆, ◇, fleroxacin.

Table I. Apparent cellular to extracellular concentration ratios of fluoroquinolones in various cells upon incubation in the presence of the corresponding drug (10 mg/l) for 30 min at 37°C. Values are given ± S.D. (n = 3–6)

Cell type	Drug pefloxacin	lomefloxacin	fleroxacin
J774 macrophages	7·7 ± 1·5	6·5 ± 0·8	5·3 ± 0·4
Human polymorphonuclear leucocytes	4·0 ± 0·1	3·8 ± 0·1	2·0 ± 0·1
Rat embryo fibroblasts	6·3 ± 0·1	6·6 ± 0·5	3·1 ± 0·3
Human erythrocytes	0·07 ± 0·01	0·7 ± 0·1	0·14 ± 0·01

the presence of 10 mg/l of the corresponding drug. Uptake proceeded rapidly and a plateau was obtained after 15–30 min of incubation. At 4°C, uptake was considerably slower for pefloxacin and lomefloxacin, and no net accumulation was seen with fleroxacin. Similar results were observed for ciprofloxacin and ofloxacin (data not shown). The cellular content of each of the five fluoroquinolones tested was directly proportional to its extracellular concentration in a 0–50 mg/l range after an incubation period of 30 min at 37°C (data not shown). Table I shows the apparent cellular accumulation of pefloxacin, lomefloxacin and fleroxacin after 30 min of incubation in three other cell types, namely polymorphonuclear phagocytes, rat embryo fibroblasts and human erythrocytes, in comparison with J774 macrophages. Accumulation could be demonstrated in all three types of nucleated cells, but not in erythrocytes. On average, at all time points and for all types of nucleated cells investigated, the cellular content of pefloxacin was similar to or greater than that of the other fluoroquinolones examined in this study.

Figure 2 shows the distribution of cell-associated fluoroquinolones (pefloxacin, lomefloxacin, fleroxacin) in J774 macrophages incubated with the corresponding drug for 30 min at 37°C, after fractionation of cytoplasmic extracts by density gradient centrifugation. For this study, macrophages were collected and homogenized, and a fraction containing nuclei and unbroken cells removed by low speed centrifugation. The resulting cytoplasmic extract, containing approximately 85% of the drug originally collected with the cells, was deposited on the top of a linear sucrose gradient. This gradient was then spun sufficiently to allow complete migration of cytoplasmic components down to the size of the smallest vesicles derived from the endoplasmic reticulum and plasma membranes ('microsomes') to their position of buoyant density, but not of the soluble constituents of the cytosol (Beaufay & Amar-Costesec, 1976). Thus, as shown in Figure 2, N-acetyl-beta-hexosaminidase, which is associated with lysosomes, moved to densities around 1·16, whereas lacticodehydrogenase, a soluble enzyme of the cytosol, was not equilibrated into the gradient but partly migrated into it, consistently with its molecular weight (approximately 130 kDa) and our conditions of centrifugation (see Lopez-Saura, Trouet & Tulkens, 1979). Proteins showed a first peak in the fractions in which lacticodehydrogenase was found (soluble proteins) and a shallow distribution throughout the remaining fractions. Cytochrome oxidase, associated with mitochondria, and inosine diphosphatase, partly associated with the pericellular membrane, moved into the gradient and equilibrated at densities clearly

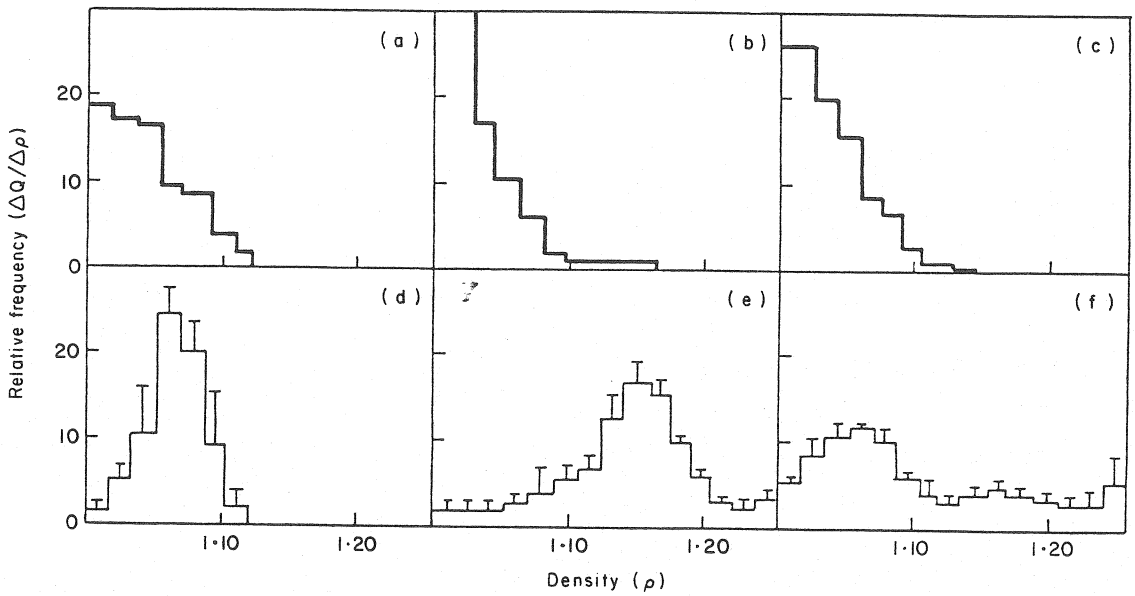

Figure 2. Distribution of cell-associated fluoroquinolones in post-nuclear supernates of homogenates prepared from J774 macrophages incubated with the corresponding drug (10 mg/l) at 37°C for 30 min, after centrifugation in sucrose gradients. The lower diagrams show the mean distributions (±S.D.) of the cell constituents assayed in the three experiments. (a) Pefloxacin; (b) lomefloxacin; (c) fleroxacin; (d) lacticode-hydrogenase; (e) *N*-acetyl-*β*-hexosaminidase; (f) proteins.

distinct from that of *N*-acetyl-beta-hexosaminidase (data not shown). In sharp contrast to the behaviour of these components, cell-associated fluoroquinolones remained largely in the top fractions of the gradient, the first three of which corresponded to the sample deposited on the gradient, and little drug moved further into the gradient.

Comparative activities of pefloxacin and roxithromycin against S. aureus *in J774 macrophages*

In the next series of experiments, we examined the activity of cell-associated pefloxacin against intracellular *S. aureus*, in comparison with roxithromycin, a macrolide with considerable accumulation in J774 macrophages (Carlier *et al.*, 1987*a*). For this purpose, J774 macrophages were allowed to phagocytose opsonized *S. aureus*, then washed and exposed to lysostaphin for 15 min to kill extracellular bacteria, and reincubated at 37°C. Table II shows that cells collected 4, 8 and 24 h after phagocytosis and lysostaphin wash contained increasing amounts of bacteria. The rate of this bacterial growth was only slightly lowered (approximately 10%) if cells were reexposed to lysostaphin for 15 min every 4 h, or if incubation was carried out in the presence of oxacillin or gentamicin at extracellular concentrations 10-fold greater than their MICs determined in broth for the strain of *S. aureus* used in these experiments. Table II also shows that the addition of pefloxacin or of roxithromycin, at extracellular concentrations equal to or greater than their MICs measured in broth, resulted in a time- and concentration-dependent decrease of the numbers of cell-associated *S. aureus*. In these experiments, performed at a pH of 7·2–7·4, roxithromycin and pefloxacin displayed essentially similar activity when compared at equipotent extracellular concentrations, except at 24 h and at the lowest concentration tested (1 × the MIC) at which pefloxacin

Table II. Influence of roxithromycin and pefloxacin on the survival of cell-associated *S. aureus* after phagocytosis by J774 macrophages and subsequent incubation at 37°C[a]

Drug	Extracellular concentration[a]	Viable, cell-associated bacteria (in % of original inoculum; ± S.D.; $n = 3$) collected after a post-phagocytosis incubation for 4 h	8 h	24 h
None		282±9	573±8	8722±48
Roxithromycin	1	87±6	66±6	64±2
	10	63±4	35±2	23±2
Pefloxacin	1	92±5	57±5	37±3
	10	60±7	25±3	19±3

[a]In multiples of the MICs measured in broth against the strain of *S. aureus* used in those experiments (roxithromycin, 0·5 mg/l; pefloxacin, 1 mg/l).

was about twice as active as roxithromycin. The intracellular accumulation of roxithromycin, however, was approximately one and a half times as great as that of pefloxacin (data not shown). We previously reported that the cellular accumulation of macrolides is decreased, whereas that of fluoroquinolones is increased, when incubation of J774 macrophages is made at acidic pH (Carlier *et al.*, 1987*a,b*). The efficacy of both types of drugs against cell associated *S. aureus* was therefore reexamined at different pHs in this model, in parallel with the measurement of the cellular accumulation of the drugs. Table III shows the results obtained after 24 h of incubation at extracellular concentrations of drugs corresponding to their MICs. The efficacy of roxithromycin against cell-associated *S. aureus* and its cellular accumulation were markedly decreased at acid pH, whereas the opposite was seen for pefloxacin. Thus, at acid pH (6), roxithromycin was incapable of reducing the cell-associated inoculum, whereas pefloxacin reduced it almost 100-fold; yet the cellular accumulation of both drugs at that pH, expressed by

Table III. Influence of pH on the cellular accumulation and antibacterial effect of roxithromycin and pefloxacin (at extracellular concentrations of 1 × MIC) on *S. aureus* associated with J774 macrophages

Drug	pH	Antibacterial efficacy[a] (A)	Cellular accumulation[b] (B)	(A/B) × 10^3
Roxithromycin	6	0·01±0.01	11·2±1·8	0·9
	7	0·12±0·01	16·6±0·8	7·2
	8	0·36±0·01	32·4±0·8	11·1
Pefloxacin	6	1·02±0·09	13·5±1·2	75·6
	7	0·56±0·01	10·9±0·2	51·4
	8	0·34±0·01	9·8±0·1	34·7

[a]Log (no. of cell-associated cfus at time = 0)/(no. of cell-associated cfus at time = 24 h).

[b]Ratio of the apparent cellular to the extracellular concentrations.

reference to their extracellular concentration, was similar. At pH 8, both drugs showed a similar activity against cell-associated *S. aureus*, but this was obtained with intracellular concentrations of roxithromycin three times those of pefloxacin. These results indicate that the intrinsic activities of pefloxacin and roxithromycin towards intracellular *S. aureus* are markedly different. This is all the more evident if antibiotic efficacy is examined by reference to the cellular accumulation, i.e. if antibiotic activity units are divided by the cellular to extracellular concentration ratio, as is also shown in Table III. Thus, at all pH values, the intrinsic efficacy of pefloxacin greatly exceeded that of roxithromycin, for equivalent amounts of cell-associated antibiotic (i.e., for amounts of cell-associated drugs having the same potency in a cell-free system, as determined by their MICs measured in broth). Actually, the difference varied from three-fold at pH 8 to 80-fold at acid pH.

Uptake, release and distribution of pefloxacin in legionella-infected macrophages

Figure 3 shows the accumulation and release kinetics of pefloxacin with unelicited peritoneal macrophages obtained from guinea pigs, either uninfected or infected with *L. pneumophila*. For the latter study, cells were exposed to opsonized *L. pneumophila* at a bacteria-to-macrophage ratio of 10:1 for 60 min at 37°C, washed extensively and reincubated for 3 h, twice, in bacteria-free medium at 37°C. Macrophages typically contained two to five bacteria, as documented morphologically and by the determination of the cfus in cell lysates (data not shown). Infection by legionella resulted in a

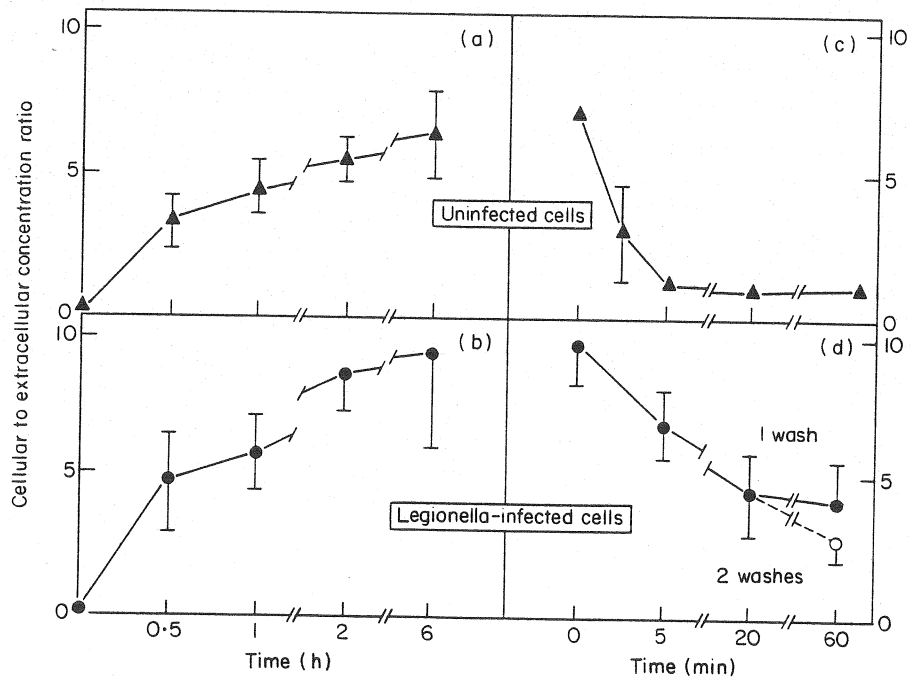

Figure 3. Accumulation and release of pefloxacin by unelicited peritoneal macrophages from guinea pigs. (a), (b) Cells were incubated in the presence of pefloxacin (10 mg/l) during the periods of time indicated in the abscissa; (c), (d) cells were incubated for 6 h in the presence of pefloxacin (10 mg/l) and then transferred to and reincubated in an antibiotic-free medium for the periods of time indicated in the abscissa. (d) One wash refers to cells treated as indicated in (c); two washes refer to cells transferred to a first antibiotic-free medium once at time = 0 min, and to a second antibiotic-free medium at time = 20 min.

larger and slightly faster accumulation of pefloxacin. Most interestingly, infection also caused a slower and largely incomplete release of the drug. The cell-associated pefloxacin partly withstood a second washing of the cells and reexposure to a second drug-free medium, suggesting that it was largely maintained in a non- or slowly-exchangeable pool, in sharp contrast to what was observed with uninfected cells, from which most of the drug readily leaked out.

We next compared the distribution of pefloxacin in post-nuclear supernatants prepared from homogenates of uninfected and legionella-infected unelicited peritoneal macrophages incubated for up to 6 h in the presence of the drug. In uninfected macrophages (data not shown), pefloxacin consistently displayed a distribution essentially similar to that observed and reported in Figure 2 for J774 macrophages, i.e. an almost complete recovery in the top fractions of the gradient where the sample had been deposited. If cells were reincubated in drug-free medium for 1 h, the very low amounts of drug remaining associated with cells (less than 8%; see Figure 3) showed a shallow distribution throughout the gradient. As depicted in Figure 4 (right diagrams), the distribution of cell-associated pefloxacin in legionella-infected macrophages was largely similar to that observed in uninfected cells or in J774 macrophages after 30 min of incubation. After 6 h, however, the distribution of pefloxacin had become unambiguously bimodal. The second mode was in fractions of larger density than those where the lysosomal enzyme *N*-acetyl-β-hexosaminidase was recovered. In post-nuclear supernates of infected cells exposed to pefloxacin for 6 h, and reincubated for 1 h in drug-free medium, the antibiotic remaining associated with cells showed a predominant distribution in the same fractions of high density. To try to determine the nature of the cytological entities migrating at those high densities, these experiments were repeated using [^{3}H]-labelled legionella, obtained by growing the bacteria in the presence of [^{3}H]-thymidine followed by a 6 h wash-out. As shown in Figure 4 (left diagrams), the distribution of ^{3}H was unimodal 30 min after phagocytosis of the labelled legionella, with an almost exclusive localization in fractions of high density. This pattern remained largely unchanged in cells incubated with the antibiotic for 6 h after phagocytosis of the bacteria, as well as in cells subjected to 1-h wash-out in drug-free medium after a 6-h contact with the antibiotic.

Discussion

The experiments reported in this paper examined the accumulation and distribution of selected fluoroquinolones, with special reference to pefloxacin, in both infected and uninfected macrophages, in relation to the activity of these agents against sensitive intracellular bacteria. Our results extend those of Easmon & Crane (1985), Easmon *et al.* (1986), Carlier *et al.* (1987*b*), 1989) and Pascual *et al.* (1989). Thus, although we confirmed that fluoroquinolones are accumulated by cells, we failed to establish unambiguously their subcellular localization. The distribution patterns observed are indeed compatible with either a true distribution of the drugs in the cytoplasm of the cell, or their reversible association with organelle(s) or constituent(s) from which they may elute during cell homogenization and/or fractionation. This is made plausible by the rapid rate of efflux of pefloxacin from uninfected macrophages. A rapid efflux from macrophages, however, was also observed for macrolides (Carlier *et al.*, 1987*a*), but it was possible to demonstrate a stable association of about half the cell-associated drug with lysosomes under conditions of fractionation similar to those used here. Thus, the

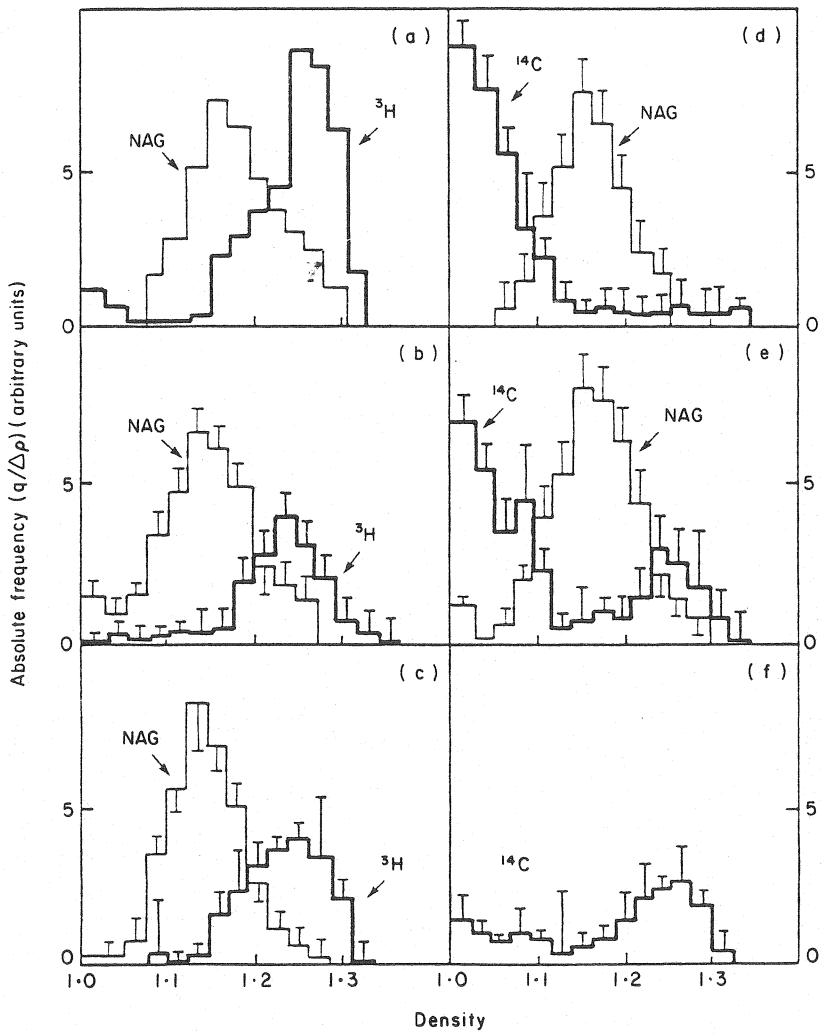

Figure 4. Distribution of [^{3}H], [^{14}C] and *N*-acetyl-β-hexosaminidase (NAG) in post-nuclear supernates prepared from unelicited peritoneal macrophages from guinea pigs and fractionated by centrifugation through sucrose gradients. (a)–(c) cells were exposed to opsonized [^{3}H]-labelled *L. pneumophila*, and then incubated in the presence of pefloxacin (5 mg/l) (30 min (a), 6 h (b), or 6 h followed by a 1 h reincubation in drug-free medium (c)). (d)–(f) A similar experimental protocol was used, except that the legionella were not labelled but [^{14}C]-labelled pefloxacin was used. Vertical bars, when shown, refer to S.D. of the distributions (n = 3).

subcellular distribution of the fluoroquinolones in the living cells cannot be ascertained on the basis of the available data.

Cell-associated fluoroquinolones retain and express a biological activity against both *S. aureus*, as shown here for pefloxacin, and by Easmon *et al.* (1986) and Carlier *et al.* (1989) for other fluoroquinolones, and *L. pneumophila* (Vildé *et al.*, 1986). Both models offer the possibility of comparing fluoroquinolones and macrolides with respect to activity and its correlation with uptake and distribution. Pefloxacin and roxithromycin showed towards *S. aureus* an activity that was correlated to their intracellular accumulations. Pefloxacin, however, seems to be intrinsically more active intracellularly, achieving a similar effect to roxithromycin with lower equipotent cellular concentrations (i.e. expressed as multiples of MICs). Incubation in acid medium enhances this

difference, since the gain in activity of pefloxacin (as well as the loss of activity of roxithromycin) appears more marked than expected on the basis of the differences in accumulation caused by pH variation. It is tempting to speculate that this results from a greater bioavailability of the fluoroquinolones and/or a lower inactivating influence of the cellular physico-chemical environment, in comparison with macrolides. Assuming that fluoroquinolones are genuinely in the cell cytosol, and since part of the macrolides is in lysosomes (Carlier *et al.*, 1987*a*), one could hypothesise that the latter localization is actually unfavourable, contrary to what has often been suggested (see, e.g., de Duve *et al.*, 1974; Tulkens, 1985). Experiments involving the simultaneous localization of the drugs and the infecting organisms, and a measure of their actual contact would be of great interest in this connection. The clinical relevance of the differences in activities between roxithromycin and pefloxacin against intracellular *S. aureus*, however, is not established, since it is clear that, at neutral pH, the lower intrinsic activity of roxithromycin will be easily compensated by an increase in extracellular concentration to still clinically-achievable values. This, however, may not be true for erythromycin, which accumulates less than roxithromycin. (Carlier *et al.*, 1987*a*), and is accordingly less active against intracellular *S. aureus* (Scorneaux & Tulkens, 1989). In acidic environments, such as in abcesses, it may also no longer be possible to compensate for the difference in activity between a fluoroquinolone and a macrolide, and it may be worthwhile to perform investigations addressing this question specifically. It may also be of interest to examine the activity of fluoroquinolones with greater accumulation than pefloxacin, since these may offer a still larger difference of overall efficacy in comparison with macrolides.

The present experiments also disclose that infection of macrophages by *L. pneumophila* increases the cellular accumulation of pefloxacin. This effect was not seen in macrophages infected by *S. aureus*; compare data in Table III, with those reported by Carlier *et al.* (1987*a*) for uninfected J774 macrophages. Most conspicuously, however, infection also results in a persistence of part of the cell-associated antibiotic. We do not know whether these effects are related to the specific localization of legionella in phagosomes; see Moulder (1985) for review. Yet, it is interesting to note that the intracellular pefloxacin retained by cells upon wash-out is found in the same fractions as those containing [^{3}H], when cells are infected with [^{3}H]-thymidine labelled bacteria. Further experiments will need to ascertain that the label is genuinely associated with intracellular legionella, and establish whether these bacteria are viable or not. We also will need to know whether pefloxacin collected in these fractions of high density is actually freely available and bioactive, or whether the drug is in a bound-form (perhaps within the bacteria). At this stage, however, it may be speculated that this long-lasting persistence of pefloxacin in legionella-infected macrophages may contribute to the overall efficacy of this drug in experimental guinea pig legionellosis (Dournon *et al.*, 1986), and, as recently suggested (Dournon, E., this Supplement), in humans.

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