Comparative Intracellular (THP-1 Macrophage) and Extracellular Activities of β-Lactams, Azithromycin, Gentamicin, and Fluoroquinolones against *Listeria monocytogenes* at Clinically Relevant Concentrations

Stéphane Carryn,* Françoise Van Bambeke, Marie-Paule Mingeot-Leclercq, and Paul M. Tulkens

Unité de Pharmacologie Cellulaire et Moléculaire, Université Catholique de Louvain, Brussels, Belgium

The activities of ampicillin, meropenem, azithromycin, gentamicin, ciprofloxacin, and moxifloxacin against intracellular hemolysin-positive *Listeria monocytogenes* were measured in human THP-1 macrophages and were compared with the extracellular activities observed in broth. All extracellular concentrations were adjusted to explore ranges that are clinically achievable in human serum upon conventional therapy. In broth, ampicillin, meropenem, and azithromycin were only bacteriostatic, whereas gentamicin, ciprofloxacin, and moxifloxacin were strongly bactericidal in a concentration-dependent manner. In cells, ampicillin, meropenem, azithromycin, and ciprofloxacin were slightly bactericidal (0.3- to 0.8-log CFU reductions), moxifloxacin was strongly bactericidal (2.1-log CFU reduction), and gentamicin was virtually inactive. The difference in the efficacies of moxifloxacin and ciprofloxacin in cells did not result from a difference in levels of accumulation in cells (6.96 ± 1.05 versus 7.75 ± 1.03) and was only partially explainable by the difference in the MICs (0.58 ± 0.04 versus 1.40 ± 0.17 mg/liter). Further analysis showed that intracellular moxifloxacin expressed only approximately 1/7 of the activity demonstrated against extracellular bacteria and ciprofloxacin expressed only 1/15 of the activity demonstrated against extracellular bacteria. Gentamicin did not increase the intracellular activities of the other antibiotics tested. The data suggest (i) that moxifloxacin could be of potential interest for eradication of the intracellular forms of *L. monocytogenes*, (ii) that the cellular accumulation of an antibiotic is not the only determinant of its intracellular activity (for fluoroquinolones, it is actually a self-defeating process as far as activity is concerned), and (iii) that pharmacodynamics (activity-to-concentration relationships) need to be considered for the establishment of efficacy against intracellular bacteria, just as they are for the establishment of efficacy against extracellular infections.

The treatment of listeriosis with conventional antibiotic therapy remains a challenge, and the intracellular forms of *Listeria monocytogenes* have proved difficult to eradicate in the absence of a fully effective immune response (4, 5, 18, 51). The classically recommended treatment for listeriosis, which is mainly based on drug sensitivities determined in broth and clinical experience, is still a combination of ampicillin (or a penem) and an aminoglycoside (28, 45). Yet, both types of drugs fail to accumulate to significant levels in cells, at least in short-term experiments (15, 34, 40). Conversely, macrolides or fluoroquinolones accumulate in cells and show activity against the intracellular forms of *L. monocytogenes* in cell culture models (13, 30, 32, 34, 42). Yet, their use has been disappointing in animal models (2, 24), and the use of ciprofloxacin has met with clinical failures (23).

These observations may suggest the absence of a true correlation between the cellular accumulation of antibiotics in cell models and their effective in vivo intracellular activities (perhaps because of modulation by a series of critical parameters such as subcellular drug bioavailability and intracellular expression of activity (30, 31, 49). Yet, it is also possible that the macrolides and fluoroquinolones that have been examined so far exhibit intrinsic activities that are too weak (i.e., have MICs that are too high, as determined for extracellular bacteria) and therefore cannot be used in vivo at concentrations that are high enough.

The availability of new fluoroquinolones with improved activities against gram-positive bacteria such as moxifloxacin has triggered us to reexamine the potential activity of this class of antimicrobials against intracellular *L. monocytogenes*. In sharp contrast to β-lactams and macrolides, fluoroquinolones indeed display marked concentration-dependent activities (8, 27). We reasoned that efficient intracellular bacterial killing could be obtained with clinically meaningful extracellular concentrations (C_e) by the combination of this improved activity and the intracellular accumulation of fluoroquinolones. It must be pointed out, however, that the basic pharmacodynamic properties of most antibiotics, including fluoroquinolones, β-lactams, and macrolides, have so far been examined only in extracellular infections. The present work therefore aimed to examine which pharmacodynamic parameter governs the activities of these antibiotics against intracellular bacteria and to determine how critical the parameter is for effective intracellular chemotherapy.

In the present study we used a model of *L. monocytogenes*-infected human THP-1 macrophages with which we previously demonstrated that bacterial multiplication occurs predominantly in the cytosol (34). Two β-lactams (ampicillin and mero-

* Corresponding author. Mailing address: Unité de Pharmacologie Cellulaire et Moléculaire, Université Catholique de Louvain, UCL 73.70 Avenue E. Mounier 73, B-1200 Brussels, Belgium. Phone: 32-2-7647375. Fax: 32-2-7647373. E-mail: stephane.carryn@facm.ucl.ac.be.

penem), two fluoroquinolones (ciprofloxacin and moxifloxacin), a macrolide (azithromycin), and an aminoglycoside (gentamicin) were systematically examined for their levels of intracellular accumulation and quantitative assessment of bacterial killing at C_es covering the range of concentrations seen in the serum of humans after administration by conventional means.

MATERIALS AND METHODS

Materials. *N*,*N*-Diacetyl–L-Lys–D-Ala–D-Ala (the substrate of the DD-carboxypeptidase) and the purified *Streptomyces* R39 DD-carboxypeptidase were kindly prepared and donated by J.-M. Frère (Centre d'Ingénierie des Protéines, Université de Liège, Liège, Belgium). Azithromycin was obtained from Pfizer Inc. (Groton, Conn.), and ciprofloxacin and moxifloxacin were from Bayer AG (Wuppertal, Germany). These products were obtained as standard powders for microbiological studies with the following potencies: azithromycin, 94.4%; ciprofloxacin, 85.0%; moxifloxacin, 98.6%. All concentrations were corrected for 100% potency. Gentamicin and meropenem, procured as Geomycin and Meronem, respectively (the brand names of the products registered for clinical use in Belgium), were kindly donated by their corresponding Belgian marketing authorization holders (Shering-Plough Belgium [Brussels, Belgium] and Astra-Zeneca [Astra Pharmaceuticals, Brussels, Belgium], respectively). Ampicillin (sodium salt) was purchased from Sigma-Aldrich Co. (St. Louis, Mo.). Cell culture media and serum were purchased from Gibco Biocult (a subsidiary of Life Science Technologies), Paisley, Scotland. Unless stated otherwise, all other reagents were purchased from E. Merck AG (Darmstadt, Germany).

Bacterial strain and determination of MICs and MBCs. Hemolysin-producing strain EGD of *L. monocytogenes* (serotype 1/2a) was obtained, maintained, and characterized exactly as described before (34). MICs were determined both in RPMI 1640 medium (supplemented with 10% decomplemented fetal calf serum and 2 mM glutamine) and in tryptic soy broth (TSB; Difco, Becton Dickinson & Co., Sparks, Md.) by an arithmetic dilution method (34). Minimal bactericidal concentrations (MBCs) were determined in TSB by geometric dilution, with the bacterial suspension set at 5×10^5 CFU/ml and by using as the endpoint the drug concentration that caused a 5-log decrease in the original inoculum.

Time and dose-kill curves studies (acellular system). Cultures in the logarithmic phase of growth ($\approx 10^9$ bacteria/ml) were centrifuged at 14,000 rpm (Eppendorf 5415 C centrifuge; Gerätgebau Eppendorf GmbH, Engelsdorf, Germany) for 1 min at 4°C. The supernatant was removed and the pelleted bacteria were resuspended at a density of 10^6 CFU per ml in TSB. Antibiotics were then added at increasing concentrations, and the numbers of viable bacteria (CFU) were determined by plate assay (tryptic soy agar; Difco, Becton Dickinson & Co.) after appropriate periods of incubation. Samples were diluted to reach a mean bacterial content of 100 to 1,000 bacteria/plate (minimum value for experiments with a residual inoculum after exposure to the maximal concentration of fluoroquinolones, 60 CFU). Enumeration was done by a semiautomated method with a Gel Doc 2000 apparatus (Bio-Rad Laboratories, Hercules, Calif.) operated with Quantity One software (Bio-Rad Laboratories) for bacterial colony counting. We checked that the amount of antibiotic carried over was insufficient to impair bacterial growth (i.e., that each time its final concentration was severalfold lower than its MIC) and took into account the dilution of the samples and the amount of fluid (50 µl) plated onto the dishes (13 ml of agar).

Cells, cell infection, and assessment of intracellular activities of antibiotics. All experiments were performed with THP-1 cells, a human myelomonocytic cell line displaying macrophagelike activity (47). The cells were maintained as described earlier (34). In brief, the cells were cultured as a loose suspension in RPMI 1640 medium supplemented with 10% decomplemented fetal calf serum and 2 mM glutamine in an atmosphere of 95% air–5% CO_2. The cells (5×10^5 cells/ml) were infected by using a fresh inoculum of *L. monocytogenes* (2.5×10^6 CFU/ml) that had been incubated for 1 h at 37°C and washed extensively to remove nonphagocytosed and non-firmly adherent bacteria (four successive sedimentations at 1,300 rpm [5810R Centrifuge; Gerateban Eppendorf, GmbH, Engeldorf, Germany] followed by gentle resuspension in prewarmed sterile phosphate-buffered saline), yielding an average infection index of one bacterium per four macrophages (as determined by counting the numbers of CFU). The cells were thereafter incubated in fresh medium (with or without antibiotics) for up to 5 h. To ensure the absence of extracellular bacteria, the culture medium that contained cells with phagocytosed *L. monocytogenes* and that had not been exposed to antibiotics after the washing procedure described here were incubated at 37°C for 48 h; no bacterial growth was detected. At suitable intervals, the cells were collected by centrifugation, washed with ice-cold sterile phosphate-buffered saline, and lysed in distilled water. The lysates were then plated on

TABLE 1. MICs and MBCs of the antibiotics used in the present study for *L. monocytogenes* EGD serotype 1/2a

Antibiotic	MIC[a] (mg/liter) in:		MBC[b] (mg/liter) in TSB
	TSB	Culture medium	
Ampicillin	0.37 ± 0.23 (8)	0.30 ± 0.12 (8)	>64
Meropenem	0.050 ± 0.000 (4)	0.072 ± 0.005 (4)	>64
Ciprofloxacin	1.36 ± 0.06 (3)	1.40 ± 0.17 (3)	4
Moxifloxacin	0.54 ± 0.05 (5)	0.58 ± 0.04 (5)	2
Azithromycin	0.37 ± 0.11 (3)	0.43 ± 0.06 (3)	>64
Gentamicin	0.77 ± 0.06 (3)	0.83 ± 0.06 (3)	4

[a] Arithmetic dilutions (0.1-µg/ml increments were used for all antibiotics except meropenem (for which [0.01-µg/ml increments were used]). Values are means ± standard deviations the number of independent determinations is given in parentheses.
[b] Geometric dilutions.

tryptic soy agar at appropriate dilutions for determination of the number of viable bacteria by counting of the colonies (determination of the numbers of CFU) and used for total cell protein measurement (29). All results are expressed as the number of CFU per milligram of cell protein.

Determination of cellular antibiotic accumulation. Ciprofloxacin and moxifloxacin concentrations were measured by a fluorimetric assay by previously described procedures (32, 39) by use of excitation and emission λ readings of 275 and 450, respectively, for ciprofloxacin and 298 and 504 nm, respectively, for moxifloxacin. A systematic correction for nonspecific fluorescence due to cell protein was performed by subtracting the values obtained for samples from cells not exposed to fluoroquinolones and using protein determination for normalization of the values between samples with different protein contents. This correction amounted to approximately 30 and 10% of the raw readings for ciprofloxacin and moxifloxacin, respectively (due to the conditions of the assay). We checked that the infection of the cells did not cause significant changes in the levels of nonspecific fluorescence. The lowest limits of detection for ciprofloxacin and moxifloxacin (in lysis medium, which consisted of 0.1 M glycine-HCl [pH 3]) were 10 and 25 ng/ml, respectively, with linearity ($R^2 = 0.997$ and 0.999, respectively) at concentrations up to 200 and 400 ng/ml, respectively. The ampicillin concentration was determined by fluorimetry of the 3,6-disubstituted piperazine formed in acid solution in the presence of formaldehyde, as described by Jusko (26) (excitation λ, 346 nm; emission λ, 422 nm; lowest limit of detection, 50 ng/ml; linearity [$R^2 = 0.992$], concentrations up to 500 ng/ml). Meropenem was assayed by an enzymatic method based on the ability of β-lactams to bind to and irreversibly inhibit the bacterial DD-carboxypeptidase (from *Streptomyces* R39), as described by Frère et al. (19) (lowest limit of detection, 1 ng/ml; linearity [$R^2 = 0.906$], concentrations up to 30 ng/ml). Azithromycin concentrations were measured by microbiological assay (by the disk diffusion method) with *Micrococcus luteus* ATCC 9341 and antibiotic medium 2 (Difco, Becton Dickinson & Co.) adjusted to pH 8 (lowest limit of detection, 0.5 µg/ml; linearity [$R^2 = 0.998$], concentrations up to 8 µg/ml). Gentamicin concentrations were also measured by microbiological assay, but *Bacillus subtilis* was used as the test organism (lowest limit of detection, 1 µg/ml; linearity [$R^2 = 0.934$], concentrations up to 256 µg/ml). The drug contents of the cells were systematically expressed by reference to the corresponding protein contents of the cells, and the apparent intracellular concentration (C_c) was determined by using a conversion factor of 5 µl of cell volume per mg of cell protein, as discussed in previous publications (43, 48).

Statistical analyses. Curve-fitting analyses were done with GraphPad Prism software (version 2.01; GraphPad Software, San Diego, Calif.). Other analyses were performed with *XL*STAT software (version 4.2; Thierry Famhy, 1995 to 1999; AddinSoft SARL, Paris, France). Comparisons between groups were done by Student's *t* test, and comparisons between curves (for data presented in Fig. 6) were done by analysis of covariance.

RESULTS

MICs and MBCs. The MICs and MBCs of the antibiotics used in the present study are shown in Table 1. No constant, major differences were observed between the MICs determined in broth and those determined in the macrophage culture medium. Apart from meropenem, which displayed a remarkably low MIC, all MICs were between 0.3 and 1.4 µg/ml,

TABLE 2. C_{max} of the antibiotics used[a] and corresponding C_{max}/MIC ratios

Antibiotic	C_{max} (mg/liter)	Human dosage yielding a similar C_{max} in serum[b] (reference)	C_{max}/MIC[c]
Ampicillin	50	1 g i.v. (17)	167
Meropenem	50	1 g i.v. (9)	694
Ciprofloxacin	4.3	750 mg p.o. (Bayer Corp.[d])	3
Moxifloxacin	4	400 mg p.o. (44)	7
Azithromycin	0.4	500 mg p.o. (16)	0.9
Gentamicin	18	5 mg/kg i.v.[e] (21)	22

[a] The C_{max} were meant to mimic the maximal concentrations obtainable in serum in vivo after the administration of commonly accepted dosages to humans.
[b] i.v., intravenously; p.o., orally.
[c] Based on the MICs observed in macrophage culture medium (see Table 1).
[d] Bayer Corporation (U.S. ciprofloxacin package insert, 2000).
[e] Once-daily dosing.

i.e., within an approximately twofold geometric dilution range. Determination of MBCs showed that both β-lactams and azithromycin were essentially bacteriostatic over a clinically meaningful range of concentrations. Conversely, both fluoroquinolones and gentamicin were bactericidal within that range.

General experimental design. Whether the experiments dealt with extracellular (in broth) or phagocytosed bacteria, all experiments were conducted for a maximum of 5 h. Typical bacterial growth in the absence of antibiotic was approximately 1.2 log CFU in broth and approximately 0.8 log CFU in cells. Antibiotic concentrations were systematically adjusted to concentrations ranging from 0.5 to 100% of the peak concentration (C_{max}) usually observed in the serum of humans after administration of conventional doses of each drug (Table 2).

Activity against extracellular *L. monocytogenes*. In a first series of experiments, we measured the rate of killing of *L. monocytogenes* in broth when the organism was exposed to a fixed antibiotic concentration corresponding to the C_{max} of each drug. The data presented in Fig. 1 show that the extent and rates of killing were very different among the different antibiotic classes. Thus, the two β-lactams (ampicillin and meropenem) and azithromycin were essentially bacteriostatic. On the contrary, gentamicin and the two fluoroquinolones (ciprofloxacin and moxifloxacin) achieved considerable bacterial killing, and this killing progressed almost linearly with time. Of interest, however, was the fact that gentamicin was immediately bactericidal, whereas a lag period of about 1 h was observed for ciprofloxacin and moxifloxacin.

To further explore the influence of the drug concentration
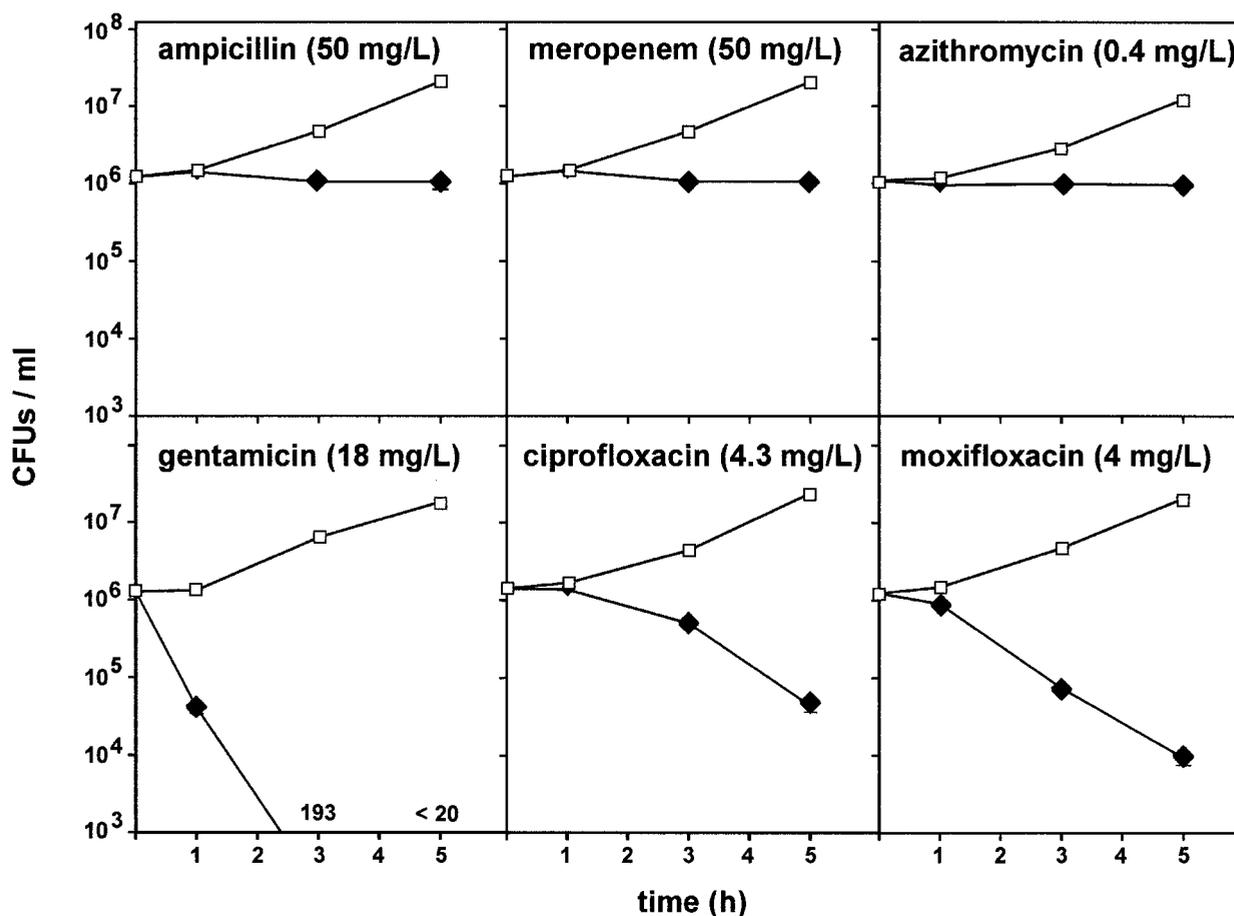

FIG. 1. Influences of antibiotics on *L. monocytogenes* survival (numbers of CFU) in broth upon incubation at a fixed drug concentration for up to 5 h. Closed diamonds, test antibiotics; open squares, controls (to which no antibiotic was added). Values are given as arithmetic means ± standard deviations (*n* = 3), but most of the corresponding error bars are smaller than the symbols. The values at the bottom of the panel for gentamicin show the number of CFU recorded at the corresponding times.
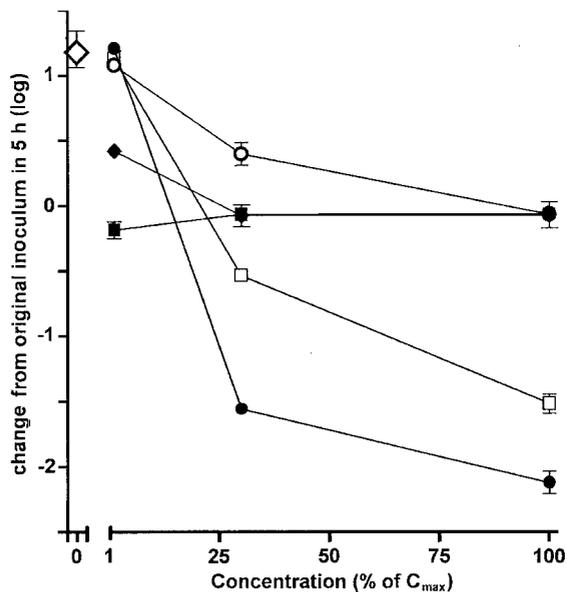
FIG. 2. Influences of antibiotics on *L. monocytogenes* survival (numbers of CFU) in broth upon incubation for a fixed period of 5 h with increasing concentrations of antibiotics (as a percentage of their corresponding C_{max}s [Table 2]). Large open diamond, value observed in controls (no antibiotic was added); closed diamonds, ampicillin (C_{max}, 50 mg/liter); closed squares, meropenem (C_{max}, 50 mg/liter); open circles, azithromycin (C_{max}, 0.4 mg/liter); open squares, ciprofloxacin (C_{max}, 4.3 mg/liter); closed circles, moxifloxacin (C_{max}, 4 mg/liter). Values are given as arithmetic means ± standard deviations (n = 3), but some of the corresponding error bars are smaller than the symbols.

on bacterial growth, the bacteria were then exposed for 5 h to drug concentrations equivalent to 1, 30, and 100% of their C_{max} (Fig. 2). It appears that meropenem achieves all of its effect at the lowest concentration tested (0.5 mg/liter; 1% of its C_{max}), with no improvement at higher concentrations. Ampicillin was maximally effective at 15 mg/liter (30% of its C_{max}). Azithromycin was considerably less potent than ampicillin or meropenem at low concentrations but eventually was as potent as ampicillin or meropenem when it was used at its C_{max} (0.4 mg/liter). All these antibiotics, however, remained bacteriostatic. In contrast, both ciprofloxacin and moxifloxacin became bactericidal once their concentrations exceeded approximately 15 to 20% of their C_{max} (i.e., concentrations close to their MICs), and this effect was clearly concentration dependent.

Activity against intracellular bacteria. In a series of experiments, we measured the activities of antibiotics against *L. monocytogenes* that had been phagocytosed by THP-1 macrophages. We first examined the influence of time using for each antibiotic a fixed C_e corresponding to its C_{max}. The results are shown in Fig. 3. Ampicillin and meropenem were slowly bactericidal, but the reduction in the numbers of CFU seen at 5 h did not exceed 0.5 to 0.7 log. Azithromycin was only very modestly bactericidal (causing a reduction of approximately 0.3 log). Gentamicin caused an almost immediate reduction of approximately 0.3 log but did not further prevent bacterial growth, which actually occurred at the same rate as it did in the controls (to which no antibiotic was added). Ciprofloxacin was slightly bactericidal over time (and actually showed a behavior essentially similar to that of meropenem). In contrast to all

other antibiotics tested, moxifloxacin achieved a marked bactericidal effect which progressed in a time-dependent fashion after a lag period of 1 h and caused a reduction of 2.3 logs from the original inoculum at 5 h.

As for the experiment performed in broth, we then systematically investigated the influences of the C_e of the antibiotics for a fixed period of 5 h. As shown in Fig. 4, all antibiotics displayed concentration-dependent effects, but the magnitudes of the effects varied very markedly among the drugs. Thus, ampicillin, meropenem, and azithromycin achieved only a fair bactericidal effect (causing reductions of 0.3 to 0.7 log) at their maximal C_e, with most of the effect of the C_e already obtained at about 15 to 25% of their C_{max}. Ciprofloxacin was clearly less potent than all other antibiotics at low concentrations, but it eventually achieved the same fair bactericidal effect as meropenem at its C_{max} (a reduction of 0.8 log). In sharp contrast to the results for all other antibiotics, moxifloxacin caused a marked bactericidal effect, causing an approximately 2-log reduction of the inoculum when it was used at a concentration about 50% of its C_{max}. No further decrease in bacterial counts was observed, however, at higher concentrations.

Because the latter data suggested an intrinsic limitation of the intracellular activities of fluoroquinolones, we examined the effect of ciprofloxacin at 15 mg/liter (i.e., a concentration that was greater than three times its C_{max}). A 1.4-log reduction of the inoculum was seen (data not shown), suggesting some sort of intracellular limitation for this fluoroquinolone as well. A linear concentration-dependent effect would indeed have yielded an approximately 4.8-log reduction (all raw counts were at least 60 to 100 CFU per plate, ruling out the possibility that we had simply reached the lowest limit of detection by our assay). Additional studies showed that these persistent bacteria did not grow in broth in the presence of 1.2 mg of moxifloxacin per liter (two times the MIC) for at least 7 days, also ruling out a potential population distribution phenomenon or the mere contamination of our cultures by resistant organisms.

Cellular accumulation of antibiotics. Table 3 shows the levels of accumulation of ampicillin, meropenem, ciprofloxacin, and moxifloxacin in cells incubated for 5 h in the presence of the drugs at fixed C_e corresponding to their C_{max}. As anticipated, the C_c of the two β-lactams remained inferior to their C_e. The two fluoroquinolones showed a fair level of accumulation (approximately eightfold). Infection did not affect these values for any of the drugs except moxifloxacin, for which an approximately 15% reduction of the level of accumulation was seen. For gentamicin and azithromycin, the microbiological assay did not prove sensitive enough for accurate determination of the intracellular content after incubation in the presence of their corresponding C_{max}. Experiments were therefore performed with uninfected cells at larger concentrations (200 mg/liter for gentamicin, 20 mg/liter for azithromycin). These studies showed a lack of accumulation of gentamicin (<1-fold), which is in contrast to the marked accumulation of azithromycin (~100-fold), which is consistent with the results of several previous studies with these antibiotics (6, 34, 48).

Effects of concentration on the extracellular and intracellular activities of fluoroquinolones. To better examine whether fluoroquinolones showed the same concentration-dependent activities against intracellular bacteria that they showed against the extracellular ones, we systematically compared the changes
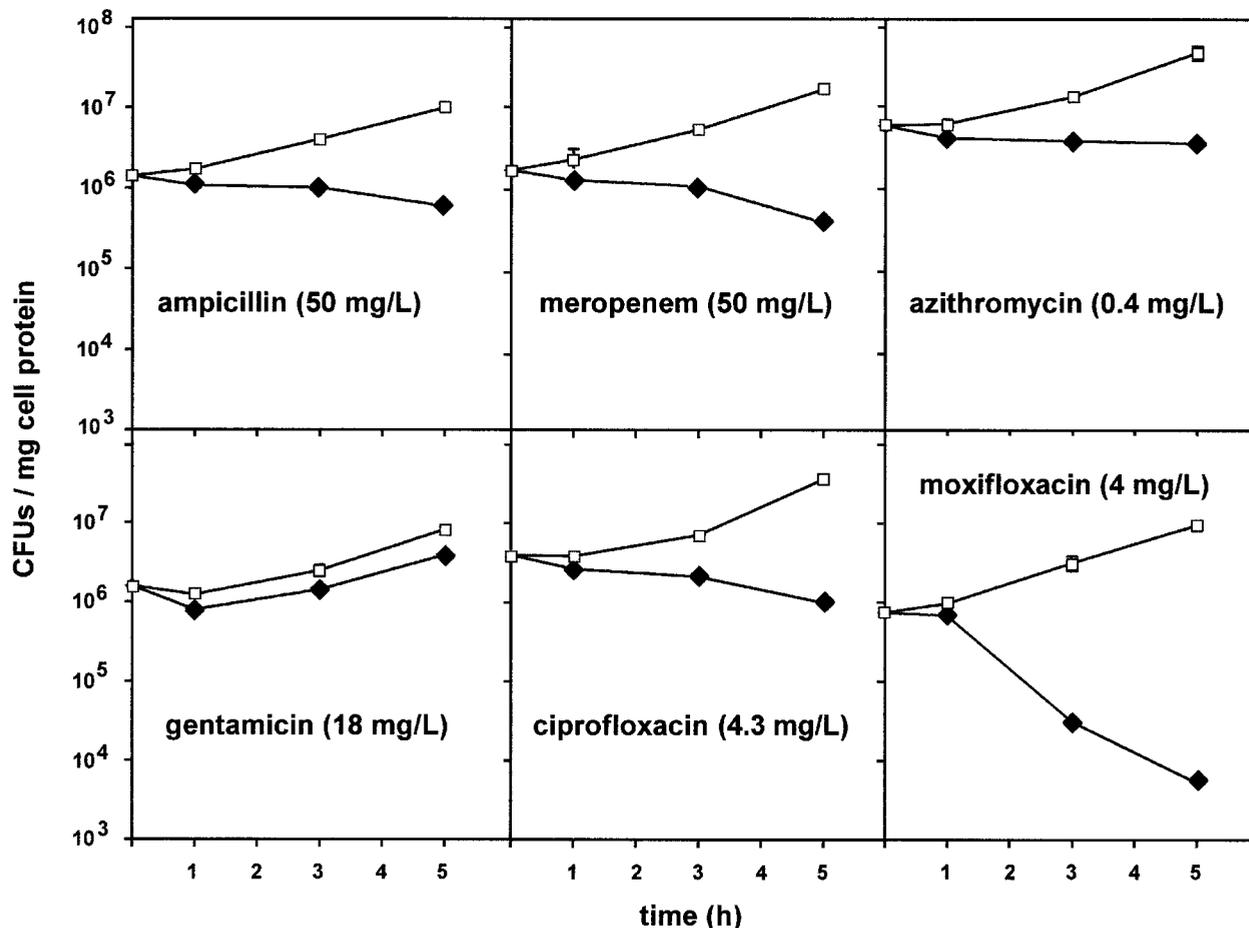
FIG. 3. Influences of antibiotics on the survival of *L. monocytogenes* (numbers of CFU) phagocytosed by THP-1 macrophages upon subsequent incubation of cells in the presence of a fixed extracellular drug concentration Closed diamonds, test antibiotics; open squares, controls (to which no antibiotic was added). Values are given as the arithmetic the mean ± standard deviation (*n* = 3) number of CFU per milligram of protein, but most of the corresponding error bars are smaller than the symbols.

in the inocula observed in broth and in cells over increasing C_e. The results are shown in Fig. 5 and demonstrate (i) that concentration dependency is indeed observed in both cases; (ii) that the concentration dependencies and maximal efficacies of ciprofloxacin and moxifloxacin in broth cannot be distinguished when the results for both drugs at the same multiples of their MICs are compared; and (iii) that, in contrast to the previous point, the concentration dependency slopes and the maximal intensities of the effects of the two drugs are quite different when they are examined against intracellular bacteria. Also quite interesting was the fact that the data show that the activities of intracellular fluoroquinolones are considerably weaker than their extracellular activities if one takes into account the fact that the drugs are concentrated in cells. To illustrate this, Fig. 6 directly compares the activities of moxifloxacin at the same C_e against extracellular and intracellular bacteria (in broth and in culture medium, respectively). The two responses are almost perfectly superimposable, as if the cellular accumulation of the drug did not result in any enhancement of activity (or as if the accumulation process is self-defeating in terms of activity). Similar data were obtained for ciprofloxacin at the same C_e (data not shown), but in that

case, the intracellular activity was about two times lower than the extracellular activity.

Influence of gentamicin on activities of other antibiotics. Because gentamicin is often combined with ampicillin or meropenem for the treatment of listeriosis, we examined whether its presence could modulate the intracellular activities of these antibiotics. The experiments were then extended to the other antibiotics used in this study (Fig. 7). As was shown earlier (Fig. 3) and as shown again for the sake of comparison, gentamicin alone at a concentration corresponding to its C_{max} caused only a modest decrease in bacterial counts (approximately 0.3 log CFU). When gentamicin was combined with ampicillin, meropenem, and azithromycin, it also increased the overall activity by about 0.3 log (additive effect). However, no significant increases in activity were seen for ciprofloxacin or moxifloxacin.

DISCUSSION

L. monocytogenes is a facultative intracellular bacterium characterized by its ability to enter various cell types, including macrophages (13, 20, 34, 50). Once it is internalized, virulent *L. monocytogenes* quickly escapes from the phagosomes
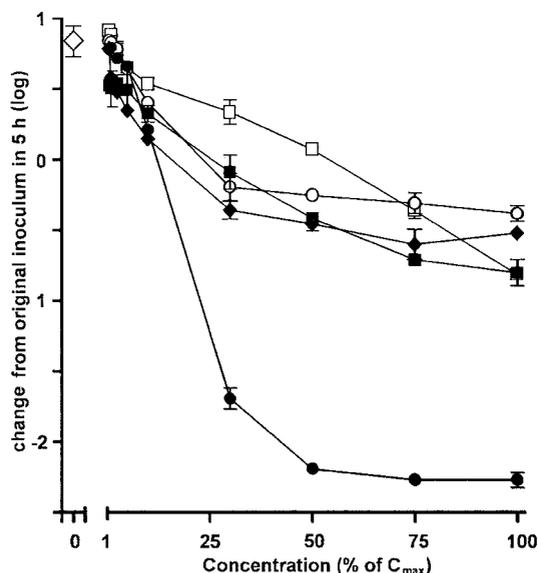
FIG. 4. Influences of antibiotics on the survival of *L. monocytogenes* (numbers of CFU) phagocytosed by THP-1 macrophages upon a subsequent incubation for 5 h at increasing drug C_e (as a percentage of their corresponding C_{max} [Table 2]). Large open diamond, value observed in controls (no antibiotic); closed diamonds, ampicillin (C_{max}, 50 mg/liter); closed squares, meropenem (C_{max}, 50 mg/liter); open circles, azithromycin (C_{max}, 0.4 mg/liter); open squares, ciprofloxacin (C_{max}, 4.3 mg/liter); closed circles, moxifloxacin (C_{max}, 4 mg/liter). Values are given as the arithmetic mean ± standard deviation ($n = 3$) number of CFU per milligram of protein.

through membrane destabilization and disruption and thrives in the cytosol (46). All these features have been observed in the model used here (34, 42), with which we also demonstrated that the escape of *L. monocytogenes* to the cytosol is almost complete after 1 h. Our model therefore essentially corresponds to a cytosolic infection and should allow us to define the conditions of the activity of an antibiotic within the cytosol. The model was further validated with respect to the main parameters that may govern intracellular activity (49), namely, the intrinsic antibacterial activities of the drugs under study

TABLE 3. Accumulation of antibiotics in macrophages and calculated C_c-to-MIC ratios[a]

Antibiotic	C_c/C_e[b]		C_c/MIC[c]
	Noninfected cells	Infected cells	
Ampicillin	0.93 ± 0.20 (12)	0.92 ± 0.19 (12)	153
Meropenem	0.96 ± 0.19 (12)	1.00 ± 0.36 (12)	694
Ciprofloxacin	7.36 ± 0.66 (6)	7.75 ± 1.03 (6)	24
Moxifloxacin	8.22 ± 0.87 (6)[d]	6.96 ± 1.05 (6)[e]	48

[a] The cells were incubated in the presence of the maximum extracellular concentrations tested in this study (ampicillin, 50 mg/liter; meropenem, 50 mg/liter; ciprofloxacin, 4.3 mg/liter; moxifloxacin, 4 mg/liter), and the levels of accumulation were determined after 5 h of incubation.

[b] The C_c-to-C_e ratio was calculated from the drug contents per milligram of cell protein by using a conversion factor of 5 μl per mg of cell protein the numbers of independent determinations are given in parentheses.

[c] Ratio of apparent C_c to the MIC (Table 1).

[d] Fluorescence assay; an independent assay with [^{14}C]moxifloxacin gave a value of 8.45 ± 1.56 ($n = 6$).

[e] $P < 0.05$ by statistical analysis by Student's *t* test (none of the other differences between uninfected and infected cells was significant).
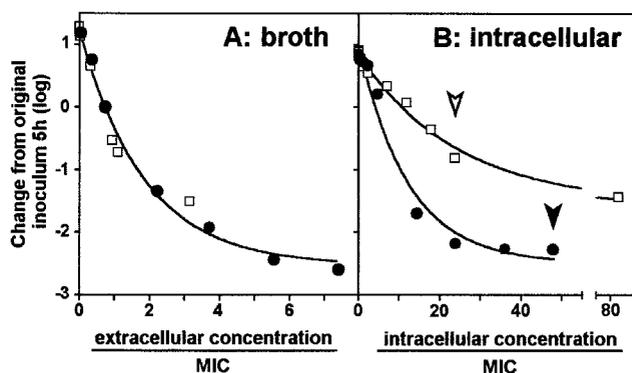

FIG. 5. Comparative extracellular (A) and intracellular (B) activities of ciprofloxacin (open squares) and moxifloxacin (closed circles) as a function of the concentration. Experiments were conducted as described in the legends to Fig. 2 and 4, except that a higher concentration of ciprofloxacin was used to achieve a maximal effect against intracellular bacteria. (A) The values on the abscissa indicate the actual drug concentration in broth; (B) the values on the abscissa indicate the calculated C_c (based on the accumulation data shown in Table 3). All values on the abscissas have been normalized with respect to the MIC of the corresponding drug in broth (A) or in the macrophage culture medium (B). Curves were generated by fitting one-phase exponential decay equations to the data (A, $R^2 = 0.9875$; B, $R^2 = 0.9793$ and 0.9858 for ciprofloxacin and moxifloxacin, respectively). The open and closed arrowheads in panel B indicate the C_c/MIC ratio reached at the C_{max} of ciprofloxacin and moxifloxacin used in all other experiments, respectively.

(i.e., their MICs and MBCs), the rates of killing, and their levels of intracellular accumulation. All values or properties were consistent with previously published data (13, 30, 31, 35, 42) or are in line with the known properties of the corresponding drugs.
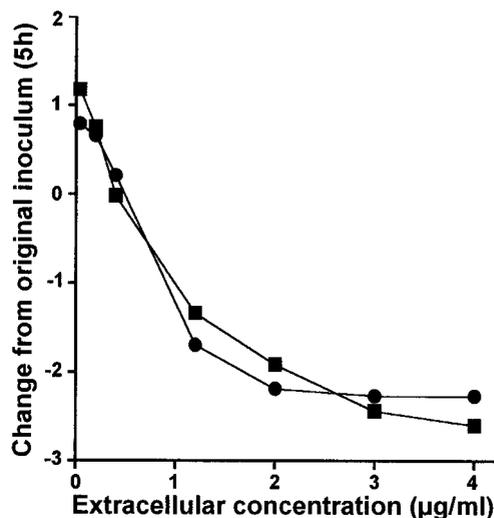

FIG. 6. Combined data for moxifloxacin from Fig. 2 and 4. The values on the abscissa indicate the actual drug concentration in broth or the macrophage culture fluid. The values on the ordinate indicate the variations in the numbers of CFU per milliliter of broth (closed squares) or per milligram of cell protein (closed circles) after 5 h of incubation. Statistical analysis (analysis of covariance) shows that there were no significant difference between the two sets of data ($P = 0.6457$).
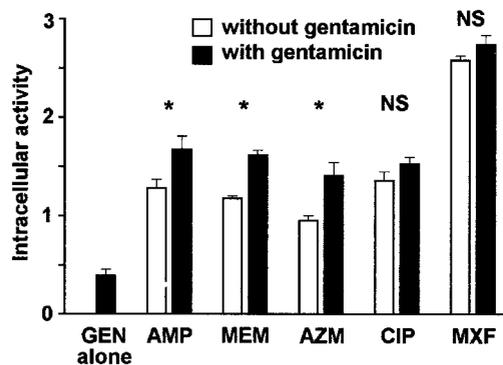
FIG. 7. Influence of gentamicin on the activities of antibiotics against intracellular *L. monocytogenes*. The cells were incubated for 5 h with the C_e of gentamicin and concentrations of the other antibiotics corresponding to their C_{max}, as follows: gentamicin (GEN), $C_{max} = 18$ mg/liter; ampicillin (AMP), $C_{max} = 50$ mg/liter; meropenem (MEM), $C_{max} = 50$ mg/liter; azithromycin (AZM), $C_{max} = 0.4$ mg/liter; ciprofloxacin (CIP), $C_{max} = 4.3$ mg/liter; moxifloxacin (MXF), $C_{max} = 4$ mg/liter. Intracellular activity (ordinate) is defined as the difference in the log bacterial counts (numbers of CFU) between cells incubated without antibiotic and (i) cells incubated with gentamicin alone, (ii) cells incubated with each of the other antibiotics alone, or (iii) cells incubated with the combination of gentamicin and the corresponding antibiotic. Each value used for the calculation was the arithmetic mean of three independent determinations. *P* values between cells exposed to an antibiotic plus gentamicin and cells exposed to an antibiotic alone, as determined by Student's *t* test, were not significant (NS) or <0.05 or less (asterisks).

When one first considers intrinsic antibacterial activity and concentrates on the MICs, it appears that meropenem should be considered the drug of choice. Yet, its lack of a bactericidal effect probably represents a major weakness in terms of eradication of the organism (note that this static effect is observed under conditions in which control bacteria are in the logarithmic phase of growth [a period of 1 to 5 h] and therefore cannot reflect only a stage of unresponsiveness following growth and dilution prior to antibiotic addition). The same applies to ampicillin, for which no bactericidal effect against extracellular bacteria could be demonstrated either.

Intracellular accumulation and subcellular localization have also been suggested to be most critical in the modulation of the activities of antibiotics against intracellular bacteria (49). The drugs used in the present study actually had quite contrasting behaviors in this respect, which can be summarized as follows. The C_c of the β-lactams never exceed the C_e (14, 34). Aminoglycosides enter cells very slowly, so that their C_cs are also systematically lower than their C_e in short-term experiments (3, 48). Fluoroquinolones show fair levels of intracellular accumulation (typically 4- to 10-fold) in all cell models (7, 37, 38). Azithromycin accumulates to a very large extent in most cells (6, 22) and achieves C_c/C_e ratios of ∼80 in THP-1 macrophages (34). All these features have been confirmed in the present study. We have also shown here (i) that meropenem, like other β-lactams, does not accumulate in cells and (ii) that the level of accumulation of moxifloxacin in THP-1 macrophages is essentially similar to that of ciprofloxacin. Concerning the subcellular distribution of antibiotics, we know that (i) β-lactams and fluoroquinolones are mostly if not exclusively localized in the cytosol (on the basis of the results fractionation

studies [7, 41]); (ii) azithromycin concentrates markedly in lysosomes, but about one-third of the cell-associated drug is also localized in the cytosol (6); and (iii) gentamicin localizes first in phagosomes (10) but is thereafter almost entirely transferred to the lysosomes (48). We see here that cells exposed to a clinically meaningful concentration of meropenem or ampicillin contain drug in an amount that should create a local concentration that largely exceeds the MIC (by a factor of ∼150 for ampicillin and by a factor of ∼700 for meropenem [Table 3]). These drugs can be considered in direct contact with their bacterial targets since both are located in the cytosol. The same reasoning applies to azithromycin, which, although it has a less favorable MIC than that of meropenem or ampicillin, largely accumulates in cells. Assuming that one-third of the cell-associated azithromycin is in the cytosol, as mentioned above, its local concentration should reach more than 30 times the MIC for *L. monocytogenes*. Thus, insufficient intracellular accumulation or inappropriate localization cannot explain the inabilities of β-lactams and azithromycin to eradicate intracellular *L. monocytogenes*. Conversely, a lack of rapid accumulation and restrictive localization are probably the factors which explain the almost complete lack of activity of gentamicin against cell-associated *L. monocytogenes*. The immediate but minor effect observed can most reasonably be ascribed to a direct action of the drug on adhering but not phagocytosed bacteria or on bacteria still in phagosomes (10).

At this point it becomes clear that the lack of concentration dependency in the intrinsic antibacterial activities of ampicillin, meropenem, and azithromycin (as determined in broth) is probably responsible for the weak eradication of intracellular *L. monocytogenes*. In other words, the reason for the failure of these drugs to eradicate intracellular *L. monocytogenes* is truly a pharmacodynamic one. This may have important implications for future research. It indeed suggests (i) that no improvement can be expected from maneuvers aimed at increasing the C_cs of β-lactams or from the selection of β-lactams with still lower MICs; (ii) that much of the eradication of intracellular *L. monocytogenes* during ampicillin or meropenem treatment is probably due to host defenses, as suggested previously for ampicillin (34) (these host defenses are probably responsible for the modest intracellular bactericidal effect seen here); and (iii) that macrolides are intrinsically unable to eradicate intracellular *L. monocytogenes*.

The situation is entirely different for the fluoroquinolones, with which, at least for moxifloxacin, we observed marked eradicating activities against intracellular *L. monocytogenes*. Several investigators have reported that fluoroquinolones are capable of killing intracellular *L. monocytogenes* (13, 30, 31, 34, 42). These studies showed that the fluoroquinolones had activities that were inversely proportional to their MICs and directly proportional to the C_e. The present study extends and systematizes these observations by (i) correlating activity to concentrations that can be obtained in human serum during conventional therapy; (ii) examining directly the relationship between drug activity and the intracellular drug concentration; and (iii) showing that moxifloxacin, in contrast to most other fluoroquinolones except clinafloxacin (30), may have real clinical potential in this context. The study also demonstrates unambiguously that one of the basic pharmacodynamic properties of fluoroquinolones, namely, the marked influence of the

drug concentration on the intensity of the killing ability, which is seen for extracellular bacteria, also exists for intracellular *L. monocytogenes*. Extension of this conclusion to other intracellular infections would be of great interest, especially if one considers noncytosolic infections. In this context, it has been shown (11, 12) that ciprofloxacin is effective against *Staphylococcus aureus* and *Mycobacterium fortuitum* in macrophages and polymorphonuclear leukocytes as soon as its C_e exceeds its corresponding MIC.

Close analysis of the data suggests, however, that additional parameters need to be considered as essential determinants in the intracellular activities of fluoroquinolones. First, and quite interestingly, we see that the intracellular accumulation of moxifloxacin does not lead to any net gain in activity, suggesting that there is some sort of intracellular defeating effect on its action, the molecular or cellular mechanism(s) of which needs to be investigated further. This defeating effect, which has also recently been described in *S. aureus*-infected THP-1 macrophages (36), may vary among fluoroquinolones (viz. ciprofloxacin versus moxifloxacin), which suggests that we can perhaps modulate it in a positive direction. Second, we have consistently observed the maintenance of a residual inoculum (0.5 to 0.8% of the original inoculum) even under conditions with maximal concentrations of moxifloxacin (as well as under conditions with maximal concentrations of ciprofloxacin, but to an even greater extent). This phenomenon, which has already been observed with extracellular bacteria (for a review, see reference 25), will require careful analysis since it may indicate that there is an intrinsic limit in the eradication properties of fluoroquinolones, the clinical significance of which, however, remains uncertain (25).

The chemotherapeutic implications of the data presented here need to be underlined. The disappointing behaviors of ampicillin and meropenem raise critical questions concerning the high rate of use of these antibiotics to treat severe cases of listeriosis and point out their probable inabilities to rapidly achieve bacterial eradication in immunocompromised patients. A poor effect of amoxicillin (0.5-log reduction of intracellular *L. monocytogenes*) has been described in a HeLa cell model at the 5-h time point (31). A progression of the decline in bacterial counts (reductions up to ~2 logs) was recorded, however, when incubation with the antibiotic was continued up to 24 h. This pattern is consistent with the known time dependency of β-lactam activities. The combination of the results of Michelet et al. (31) and the present data suggests that β-lactams will be efficient in vivo only if sustained concentrations are maintained over prolonged periods of time. These issues are now open to exploratory studies with more dynamic models. The present report also strongly suggests that azithromycin will be intrinsically poorly effective for the treatment of listeriosis, despite its intracellular accumulation to high concentrations. Available data from studies with animals suggest that this conclusion probably extends to other macrolides and even ketolides (33). We also show that gentamicin affects the intracellular growth of *L. monocytogenes* only in a minor fashion and that it offers no real synergy with β-lactams. This reinforces the conclusion that the use of gentamicin for the treatment of listeriosis can be justified only by its action against extracellular bacteria (the lack of an additive effect of gentamicin in combination with fluoroquinolones does not indicate antagonism; it more prob-

ably results from the fact that fluoroquinolones at their C_{max} are quickly bactericidal against extracellular bacteria, so that there is not much to gain by the association in this context).

Finally, like other investigators (13, 30, 31, 34, 42), we suggest that fluoroquinolones may represent a valuable option for the treatment of listeriosis, especially even in difficult situations such as meningitis, because of their marked bactericidal activities. Although the concentrations of fluoroquinolones in cerebrospinal fluid are only 20 to 50% of the concentrations in serum (1), our data show that this level already allows almost a maximal effect both extra- and intracellularly (Fig. 5). More data, however, are needed concerning the penetration of moxifloxacin into brain tissues under conditions of inflamed meninges. Nevertheless, the present data open interesting perspectives and set up the conditions for the performance of meaningful trials of moxifloxacin with animals.

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