ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1992, p. 1053–1056
0066-4804/92/051053-04$02.00/0

Vol. 36, No. 5

Uptake and Intracellular Activity of Sparfloxacin in Human Polymorphonuclear Leukocytes and Tissue Culture Cells

ISABEL GARCÍA, ALVARO PASCUAL,* M. CARMEN GUZMAN, AND EVELIO J. PEREA
Department of Microbiology, School of Medicine, Apdo 914, 41080-Sevilla, Spain

Received 8 August 1991/Accepted 20 February 1992

The penetration of sparfloxacin into human neutrophils (PMN) and different tissue culture cells (HEp-2 and McCoy) was evaluated. The cellular to extracellular concentration ratios (C/E) of sparfloxacin were always higher than 4 at extracellular concentrations ranging from 0.5 to 25 mg/liter. The uptake of sparfloxacin by PMN was rapid, nonsaturable, reversible, not energy dependent, and significantly reduced at pH 8. The penetration of this agent into PMN was similar when viable and Formalin-killed cells were used and was not affected by environmental temperature. Ingestion of opsonized zymosan significantly increased the amount of PMN-associated sparfloxacin. Sparfloxacin at a concentration of 0.5 mg induced a significant reduction in the survival of intracellular *Staphylococcus aureus*. It is concluded that sparfloxacin reaches intracellular concentrations within leukocytic cells much higher than extracellular concentrations, while remaining active intracellularly.

Most of the new quinolones have shown high intracellular penetration in different phagocytic and nonphagocytic cells. Moreover, these antimicrobial agents have been shown to be effective agents against typical facultative intracellular pathogens such as *Legionella* spp. and against other bacteria, such as *Staphylococcus aureus*, that in certain circumstances are able to survive and even multiply within phagocytic cells (2, 3, 11).

Nevertheless, most quinolones have limited activity against gram-positive cocci. Sparfloxacin is a new quinolone with two fluorinated substituents that has been associated with greater activity against gram-positive cocci than other quinolones (7). It was found to be more active against clinical isolates of *S. aureus*, including quinolone-resistant *S. aureus*, than the existing quinolones (8). Moreover, sparfloxacin offers an increased half-life and good tissue distribution (9).

The purpose of this study was to evaluate the uptake of sparfloxacin by human polymorphonuclear leukocytes (PMN) and tissue culture epithelial cells. The possible mechanism involved in the penetration of this quinolone into phagocytic cells and its intracellular activity against *S. aureus* was also evaluated.

MATERIALS AND METHODS

Isolation of PMN. PMN were recovered from heparinized venous blood of healthy donors by dextran sedimentation and Ficoll-Hypaque gradient as previously described (15). PMN preparations were 97% pure. Final cell suspensions were adjusted to 5×10^6 PMN per ml in Hanks balanced salt solution. The PMN were 95% viable by trypan blue exclusion.

Tissue culture cells. HEp-2 and McCoy cells (Flow Laboratories, Irvine, United Kingdom) were grown in minimal essential medium (Flow) supplemented with 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Flow) and containing 10% fetal calf serum (Flow) without antibiotics. For each experiment, cells were detached from tissue culture bottles with trypsin-EDTA (Flow), washed once with minimal essential medium containing fetal calf serum (10%), and suspended in Hanks balanced salt solution at a concentration of 5×10^6 cells per ml.

Sparfloxacin uptake by phagocytes. Uptake of radiolabeled sparfloxacin by phagocytes was determined by means of a velocity gradient centrifugation technique described by Klempner and Styrt (6). [^{14}C]sparfloxacin (21.4 μCi/mg) and sparfloxacin were kindly supplied by Rhone-Poulenc (Paris, France). In these experiments, PMN or tissue cells were incubated in Hanks balanced salt solution containing different concentrations of sparfloxacin (0.5, 1, 2, 5, 10, and 25 mg/liter). After different incubation periods at 37°C, cells were separated from extracellular solution by centrifugation through a water-impermeable silicone oil barrier in a microcentrifuge tube. A 10-μl aliquot of the extracellular medium and the entire cell pellet, obtained by cutting off the portion of the microfuge tube containing the pellet, were placed in 3 ml of scintillation fluid (Ready Micro, Beckman Instruments, Inc., Fullerton, Calif.) and counted in a liquid scintillation counter (model LS 1801; Beckman). After determination of the cell volume with radiolabeled polyethylene glycol and water (New England Nuclear), the accumulation rate of the antimicrobial agent in PMN or tissue cells (cellular to extracellular concentration [C/E] ratio) was calculated as described previously (6).

Characterization of sparfloxacin uptake. Further studies to elucidate the mechanism of sparfloxacin uptake by PMN were performed as described previously (4). The importance of cell viability was studied by using PMN killed by exposure to 10% Formalin for 30 min. These cells were then washed and suspended in fresh medium. Moreover, the influences of environmental temperature, pH, metabolic inhibitors, and potential competitive inhibitors were evaluated. The influence of temperature was examined by comparing antimicrobial uptake at 4 and 37°C. The pH profiles of sparfloxacin uptake in media preadjusted to different external pHs (pHs 6, 7, and 8) by the addition of 10 N HCl or 10 N NaOH were measured. Sodium fluoride (Sigma Chemical Co., St. Louis, Mo.) and sodium cyanide (Sigma) were used as metabolic inhibitors at 10^{-3} and 5×10^{-3} M. The PMN in Hanks

* Corresponding author.

balanced salt solution with or without metabolic inhibitors were incubated for 30 min at 37°C. Sparfloxacin at a final concentration of 2 μg/ml was then added, and the uptake was measured as described above. Other substances were evaluated for possible competitive inhibition of sparfloxacin uptake. These materials included a nucleoside (adenosine [Sigma]), D-glucose (Sigma), and an L-amino acid (lysine monohydrochloride [E. Merck AG, Darmstadt, Germany]). These substances were added to the cells at the same time as sparfloxacin. The uptake of sparfloxacin was measured as described above. Cell viability was always measured after each experiment by trypan blue exclusion and was always higher than 95%.

The efflux, or reversibility of binding, of PMN-associated sparfloxacin was also studied. The PMN were incubated for 20 min at 37°C with sparfloxacin, collected by centrifugation, and rapidly suspended in sparfloxacin-free medium. PMN-associated sparfloxacin was quantified at various intervals (5, 10, and 20 min) after removal of the extracellular sparfloxacin.

In a series of experiments, sparfloxacin (extracellular concentration, 2 mg/liter) uptake by human PMN was measured after stimulation of the cells with 200 nM phorbol myristate acetate (Sigma), opsonized zymosan (0.9 mg/liter; Sigma), and S. aureus ATCC 25923 opsonized in 5% pooled human serum (15 min, 37°C) at a 10/1 ratio of bacteria to PMN. Phorbol myristate acetate, zymosan, or opsonized bacteria were added to the PMN suspensions at the same time as the antimicrobial agents, and the uptake was measured as described above.

Partition coefficients of quinolones. The partition coefficients of quinolones were determined by the modified method of Nikaido (10). Solutions (10 mg/liter) of quinolones were made in 0.1 M phosphate buffer (pH 7.2). After being shaken with an equal volume of *p*-xylene at 25°C for 48 h and centrifuged at 1,870 × *g* for phase separation, the concentrations of quinolones in the aqueous phase were determined by a spectrophotometric assay measuring the A_{292} for sparfloxacin and the A_{294} for ofloxacin (Hoechst). The partition coefficients were expressed as the ratio of the amount of the compound in the *p*-xylene to that in the aqueous phase.

Intracellular activities of antimicrobial agents. To evaluate the intracellular activities of antimicrobial agents, a previously described method was used (14). Briefly, 0.1 ml of opsonized bacterial suspension (5×10^7 CFU/ml) and 0.1 ml of PMN (5×10^6 PMN per ml) were combined in a series of polypropylene biovials (Beckman) and incubated in a shaker (250 rpm) for 60 min at 37°C. After incubation, extracellular bacteria were removed by differential centrifugation. Cells were then suspended in 0.2 ml of RPMI medium (GIBCO, United Kingdom). At this time, different concentrations of sparfloxacin were added, and the vials were incubated in a shaker (250 rpm) at 37°C. Vials were removed at time zero and after 3 h of incubation (control and samples with antimicrobial agents). Cells were lysed in distilled water, and samples were diluted and pour plated in agar. Colonies were counted after 24 h of incubation at 37°C. The data were expressed as percentages of staphylococci surviving compared with levels in controls (without antimicrobial agents) at 3 h. In addition to determining bacterial survival, morphologic studies were also routinely performed at time zero and after 3 h of incubation to evaluate the disposition of bacteria (cell associated or extracellular). Samples (50 μl) were removed from biovials and deposited on glass slides. After being stained with Wright stain, samples were examined by light microscopy. All assays were performed in duplicate with PMN from five different donors.

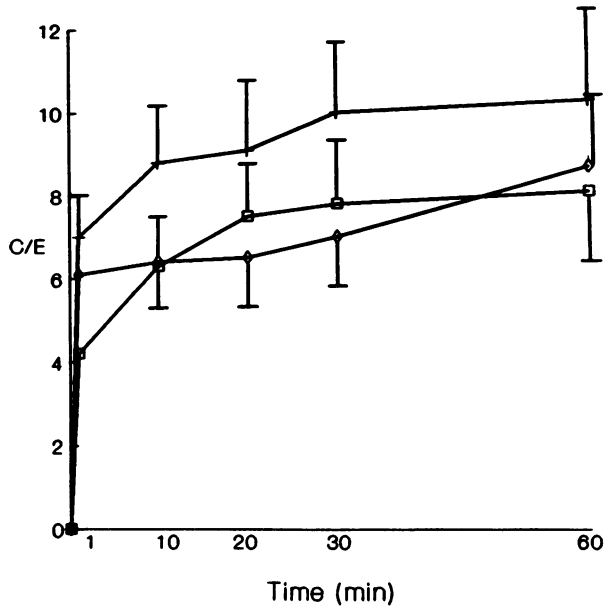

FIG. 1. Sparfloxacin uptake by human PMN (□) and two tissue culture epithelial cell lines (HEp-2 [◇] and McCoy [–]) (n = 7). The extracellular concentration was 2 mg/liter.

Statistical analysis of data. Data were expressed as the means ± standard deviations. Differences among groups were compared by analysis of variance, which was used to assess statistical significance at $P \leq 0.05$.

RESULTS

Uptake of sparfloxacin by PMN and tissue culture cells. Figure 1 shows the kinetics of the uptake of sparfloxacin by PMN and two tissue culture epithelial cell lines. Sparfloxacin uptake by these cells was rapid and high. With extracellular concentrations of 2 mg/liter, the C/E ratios were higher than 4 after only 1 min of incubation for all types of cells.

The effect of the extracellular concentration of sparfloxacin on its uptake by human PMN is shown in Fig. 2. Cell-associated sparfloxacin was not saturable at extracellular concentrations ranging from 0.5 to 25 mg/liter.

The effects of environmental temperature, pH, cell viability, and metabolic inhibitors on the intracellular penetration of sparfloxacin are described in Table 1. The uptake of sparfloxacin was not different when viable PMN at 4°C or Formalin-killed cells at 37°C were used. Sparfloxacin uptake by PMN was slightly higher at pH 6 and significantly decreased ($P < 0.05$) at pH 8. Neither sodium fluoride nor sodium cyanide affected the intracellular penetration of this agent.

Potential competitive substrates described for other antimicrobial agents (adenosine, glucose, and L-amino acids) were evaluated. The presence of adenosine significantly impaired the rate of uptake of sparfloxacin by human PMN (C/E ratio, 4.7 ± 1.4; $P < 0.05$ compared with controls [6.7 ± 1.3]). The C/E ratios for glucose and L-lysine were 7.4 ± 1.9 and 8.0 ± 2.0, respectively.

The stimulation of PMN by a membrane activator (phorbol myristate acetate) and the phagocytosis of opsonized *S. aureus* did not affect the intracellular penetration of spar-

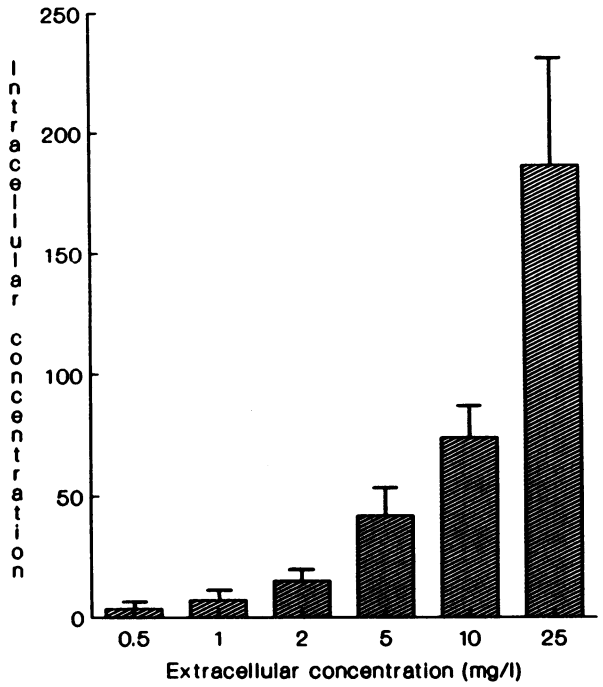

FIG. 2. Sparfloxacin uptake by human PMN at different extracellular concentrations (n = 9). Incubations were for 20 min.

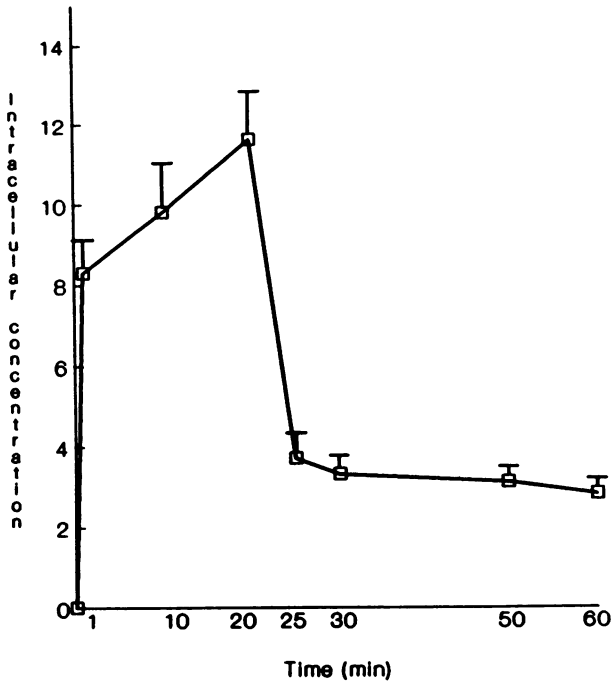

FIG. 3. Efflux of intracellular sparfloxacin from human PMN after removal of extracellular drug (n = 3). Cells were centrifuged and suspended in drug-free medium after 10 min of sparfloxacin accumulation.

floxacin (C/E ratios of 5.9 ± 1.3 and 6.0 ± 2.1, respectively). The phagocytosis of opsonized zymosan, however, significantly increased the uptake of sparfloxacin by PMN (C/E ratio of 12.3 ± 2.8; $P < 0.05$).

To evaluate whether sparfloxacin that had been taken up by PMN was tightly bound to cellular components, we studied the kinetics of efflux (Fig. 3). The reversibility of binding of sparfloxacin was rapid, with 60% of the cell-associated drug being lost by 5 min (compare values at 20 and 25 min in Fig. 3).

Partition coefficients of quinolones. The partition coefficients of sparfloxacin and ofloxacin were 0.250 and 0.090, respectively. This means that sparfloxacin is approximately four more times hydrophobic than ofloxacin.

Intracellular activity of sparfloxacin. The intracellular activities of different concentrations of sparfloxacin against *S. aureus* are shown in Fig. 4. At extracellular concentrations of 0.5 and 1 mg/liter, sparfloxacin produced a significant reduction in the survival rate of *S. aureus* in PMN.

TABLE 1. Effects of cell viability, environmental temperature, pH, and metabolic inhibitors on the intracellular penetration of sparfloxacin in human PMN (n = 5)

Condition[a]	C/E ratio
Viable cells, 37°C	6.5 ± 1.2
Viable cells, 4°C	5.3 ± 1.3
Dead cells, 37°C	6.1 ± 1.5
pH 6	7.9 ± 2.4
pH 7	6.4 ± 1.1
pH 8	3.9 ± 1.1[b]
NaF	6.6 ± 2.8
NaCN	6.2 ± 1.4

[a] Experiments were carried out for 20 min at an extracellular concentration of 2 mg/liter.

[b] $P < 0.05$ compared with the control.

DISCUSSION

In this study, the uptake of radiolabeled sparfloxacin by human PMN and tissue culture cells has been evaluated. Using radiolabeled sparfloxacin, we have observed that this agent is highly concentrated in both types of cells even at therapeutical concentrations. The C/E ratios of sparfloxacin for PMN were similar to those observed for ciprofloxacin, ofloxacin, and other quinolones by means of a fluorometric assay (11). The intracellular penetration of sparfloxacin into tissue epithelial cells and fibroblasts was, however, significantly higher than that observed for ofloxacin (12). This could be related to the fact that sparfloxacin is more hydrophobic than ofloxacin and theoretically could pass more easily through the cell membrane.

We have previously described a sensitive fluorometric assay, which avoids the use of radiolabeled antimicrobial agents, to measure intracellular concentrations of quinolones (11, 12). Nevertheless, although sparfloxacin contains two fluor substituents in its molecule, it did not emit any fluorescence under several experimental conditions.

The uptake of sparfloxacin by PMN was rapid, nonsaturable (at extracellular concentrations between 1 and 25 mg/liter), reversible, and not energy dependent. It was slightly increased at acidic pH but significantly reduced at pH 8. This phenomenon could be related to the fact that all the quinolones display a free carboxyl group which at pH 8 should be in its unprotonated form (—COO^-). It has been observed that the ionized forms of weak organic acids and bases diffuse through biological membranes much more slowly

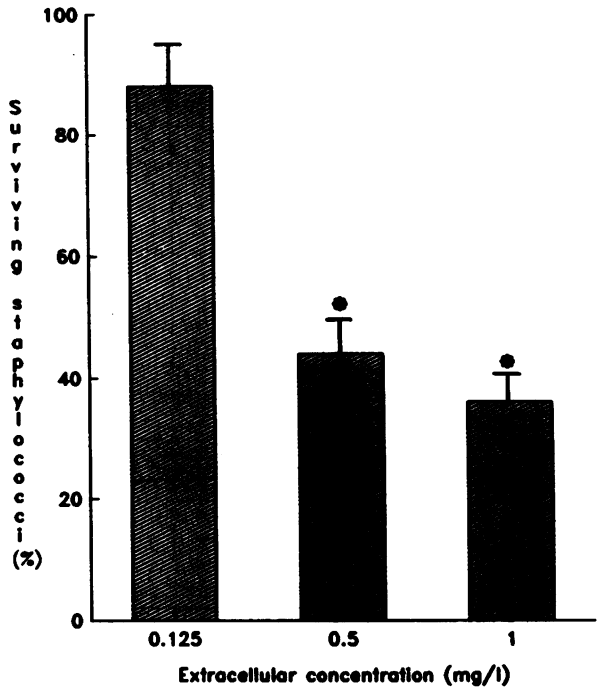

FIG. 4. Effect of sparfloxacin on survival of *S. aureus* ATCC 25923 in human PMN ($n = 5$). Data are expressed as percentages of levels in control (without antimicrobial agent). The MIC and MBC for this strain were 0.125 and 0.25 mg/liter, respectively. *, $P < 0.05$.

than their un-ionized forms (16). The intracellular penetration of sparfloxacin in human PMN was not affected by environmental temperature and cell viability. All these data point out that the uptake of sparfloxacin by PMN occurs by a passive mechanism. Similar results were described by Carlier et al. for murine macrophages (1).

The influences of phagocytosis and cell membrane stimuli on the uptake of sparfloxacin were of interest. An unexpected finding for which we do not have a clear explanation yet was that ingestion of opsonized *S. aureus* did not affect the intracellular penetration of sparfloxacin, but the ingestion of opsonized zymosan significantly increased the C/E ratios of sparfloxacin. Nevertheless, the uptake of both antimicrobial agents was still high in both conditions.

Another interesting finding is the effect of adenosine on the intracellular penetration of sparfloxacin. There are some antimicrobial agents such as clindamycin which are transported into human PMN via the nucleoside transport system (12). This mechanism, however, is active and requires energy. The mechanism whereby sparfloxacin accumulates in cells is not yet known, and no simple model can be presented in view of the findings presented above. Although most data point toward a passive mechanism, some are typical of an active mechanism. More than one simple mechanism may be involved. In fact, not all the quinolones show the same mechanism for intracellular penetration. Our previous data pointed to an active mechanism for ofloxacin (11). Moreover, sparfloxacin is much more hydrophobic than ofloxacin, a characteristic that theoretically allows easier penetration through cell membranes. Nevertheless, further studies are being performed to elucidate this point.

A contrast between phagocyte antimicrobial agent uptake and intracellular activity has been described for a few antimicrobial agents (5). In our study, sparfloxacin showed good intracellular activity against *S. aureus* in PMN. These results were similar to those described for other quinolones (13).

In summary, sparfloxacin penetrates into human PMN and tissue culture cells, reaching intracellular concentrations several times higher than extracellular concentrations mainly by a passive mechanism while remaining active intracellularly. The high antimicrobial activity of this compound added to the properties observed in this study enlarges the potential clinical use of sparfloxacin.

ACKNOWLEDGMENTS

We thank K. Allison and P. Hidalgo for preparation of the manuscript.

REFERENCES

1. **Carlier, M. B., S. Faraji, and P. M. Tulkens.** 1990. Uptake and subcellular localization of sparfloxacin (AT 4140, RP 64206; S) in phagocytic cells, abstr. 1243. Program Abstr. 30th Intersci. Conf. Antimicrob. Agents Chemother.
2. **Gay, J. D., D. R. DeYoung, and G. D. Roberts.** 1984. In vitro activities of norfloxacin and ciprofloxacin against *Mycobacterium tuberculosis*, *M. avium* complex, *M. chelonei*, *M. fortuitum*, and *M. kansasii*. Antimicrob. Agents Chemother. **26:**94–96.
3. **Greenwood, D., and A. Laverick.** 1983. Activities of newer quinolones against *Legionella* group organisms. Lancet **ii:**279–280.
4. **Hand, W. L., and N. L. King-Thompson.** 1982. Membrane transport of clindamycin in alveolar macrophages. Antimicrob. Agents Chemother. **21:**241–247.
5. **Hand, W. L., and N. L. King-Thompson.** 1986. Contrast between phagocyte antibiotic uptake and subsequent intracellular bactericidal activity. Antimicrob. Agents Chemother. **29:**135–140.
6. **Klempner, M. S., and B. Styrt.** 1981. Clindamycin uptake by human neutrophils. J. Infect. Dis. **144:**472–475.
7. **Kojima, T., M. Inoue, and S. Mitsuhashi.** 1989. In vitro activity of AT-4140 against clinical bacterial isolates. Antimicrob. Agents Chemother. **33:**1980–1988.
8. **Kojima, T., M. Inoue, and S. Mitsuhashi.** 1990. In vitro activity of AT-4140 against quinolone- and methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **34:**1123–1127.
9. **Nakamura, S., N. Kurobe, M. Hashimoto, and M. Shimizu.** 1990. Pharmacokinetics of a novel quinolone, AT-4140, in animals. Antimicrob. Agents Chemother. **34:**89–93.
10. **Nikaido, H.** 1976. Outer membrane of *Salmonella typhimurium*: transmembrane diffusion of some hydrophobic substances. Biochim. Biophys. Acta **433:**118–132.
11. **Pascual, A., I. García, and E. J. Perea.** 1989. Fluorometric measurement of ofloxacin uptake by human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. **33:**653–656.
12. **Pascual, A., I. García, and E. J. Perea.** 1990. Uptake and intracellular activity of an optically active ofloxacin isomer in human neutrophils and tissue culture cells. Antimicrob. Agents Chemother. **34:**277–280.
13. **Pascual, A., L. Martínez-Martínez, and E. J. Perea.** 1989. Effect of ciprofloxacin and ofloxacin on human polymorphonuclear leukocyte activity against staphylococci. Chemotherapy (Basel) **35:**17–22.
14. **Pascual, A., D. Tsukayama, J. Kovarik, G. Gekker, and P. K. Peterson.** 1987. Uptake and activity of rifapentine in human peritoneal macrophages and polymorphonuclear leukocytes. Eur. J. Clin. Microbiol. **6:**152–157.
15. **Peterson, P. K., J. Verhoef, D. Schmeling, and P. G. Quie.** 1977. Kinetics of phagocytosis and bacterial killing by human polymorphonuclear leukocytes and monocytes. J. Infect. Dis. **136:**502–509.
16. **Renard, C., H. C. Vander Haegue, P. J. Claes, A. Zenebergh, and P. M. Tulkens.** 1987. Influence of conversion of penicillin G into a basic derivative on its accumulation and subcellular localization in cultured macrophages. Antimicrob. Agents Chemother. **31:**410–416.