ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1989, p. 653–656
0066-4804/89/050653-04$02.00/0

Vol. 33, No. 5

Fluorometric Measurement of Ofloxacin Uptake by Human Polymorphonuclear Leukocytes

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Received 26 October 1988/Accepted 27 January 1989

A fluorometric assay, based on the natural fluorescence of the quinolone nucleus, was used to determine the uptake of ofloxacin by human polymorphonuclear leukocytes. The ratio of cellular concentration to extracellular concentration (*C/E*) at 20 min and 37°C was 7.2, using an extracellular concentration of 5 μg/ml. Uptake was rapid and was not affected by pH (5 to 9), but required elevated environmental temperature and cell viability. The metabolic inhibitors sodium fluoride and sodium cyanide significantly decreased the uptake of ofloxacin. The penetration of ofloxacin was not affected by the presence of glucose or adenosine, but was decreased by L-amino acids (lysine, leucine, and glycine). These results suggest that ofloxacin could be transported via an amino acid transport system and that the fluorometric assay is a useful method for determining the intracellular penetration of fluoroquinolones, avoiding the use of radiolabeled antimicrobial agents.

The new quinolones have been shown to be very active against different intracellular pathogens such as mycobacteria and *Legionella pneumophila* (4, 5). These antimicrobial agents are able to penetrate into phagocytes and remain active intracellularly (2, 3, 9, 12).

Quinolone penetration into phagocytes has been evaluated by high-performance liquid chromatography (HPLC) (8), radiometry (14; M. B. Carlier, B. Scorneaux, A. Zenebergh, and P. M. Tulkens, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 622, 1987), and bioassay (2, 13). The preparation of samples for HPLC requires several manipulations, large volumes of cells, and high concentrations of the antimicrobial agent. Radiometric assays are sensitive and accurate but require radiolabeled quinolones, which are not always available. Bioassay techniques are less sensitive and generally inappropriate for kinetic studies.

In this study a fluorometric assay, based on the natural fluorescence of the quinolone nucleus, was used to evaluate the uptake of ofloxacin by human polymorphonuclear leukocytes (PMN). The possible mechanism involved in the membrane transport of this antimicrobial agent is also evaluated.

MATERIALS AND METHODS

Isolation of PMN. PMN were recovered from heparinized venous blood of healthy donors and were purified by previously described methods (10). PMN preparations were 97% pure. Final cell suspensions were adjusted to 5×10^6 PMN/ml in Hanks balanced salt solution. The PMN were 95% viable by trypan blue exclusion.

Ofloxacin uptake by human PMN. A previously described fluorometric assay to measure quinolone uptake by bacterial cells was adapted to measure ofloxacin uptake by human PMN (1). Ofloxacin was kindly supplied by Hoescht AG, Frankfurt, Federal Republic of Germany. In these experiments 2.5×10^6 PMN were incubated in 0.5 ml of Hanks balanced salt solution containing clinically relevant concentrations of ofloxacin (2 and 5 μg/ml). After different incubation times at 37°C, cells were separated from extracellular solution by centrifugation through a water-impermeable silicone-oil barrier in a microcentrifuge tube (7). The entire cell pellet, obtained by cutting off the portion of the microcentrifuge tube containing the pellet, was placed in 2 ml of 0.1 M glycine-HCl buffer (pH 3.0) and agitated vigorously in a vortex shaker. Incubation for 2 h at room temperature was sufficient to release fully PMN-associated antibiotic. The samples were centrifuged for 5 min at 5,600 × *g*, and the amount of antibiotic was determined by fluorescence emission of the supernatant with a fluorescence spectrophotometer (SFM 25; Kontron Instruments, Zurich, Switzerland). The fluorescence excitation and emission maxima of ofloxacin in 0.1 M glycine-HCl (pH 3.0) were 292 and 496 nm, respectively. Controls without antimicrobial agents were always used to determine the background fluorescence emitted by PMN, and this value was subtracted from these obtained with the samples containing ofloxacin.

The intracellular water space was measured by using tritiated water and the extracellular marker [^{14}C]polyethylene glycol (^3H_2O, 1.0 mCi/g; [^{14}C]polyethylene glycol, 1.4 mCi/g [Du Pont, NEN Research Products, Boston, Mass.]). The PMN were incubated with these radiolabeled compounds for 2 min at 37°C, and then cells were separated from the extracellular fluid by velocity gradient centrifugation as described above and counted in a liquid scintillation counter. The total water content of the PMN pellet was corrected for trapped extracellular water, i.e., polyethylene glycol space, to obtain the intracellular water space. From the values obtained from this procedure, PMN-associated antimicrobial concentrations were calculated and expressed as a ratio of the cellular concentration to extracellular concentration (*C/E*) (7).

Characterization of antibiotic uptake. Further studies were performed to elucidate the mechanism of ofloxacin uptake by PMN as described previously (6). The effect of cell viability was studied by using PMN killed by exposure to 10% Formalin for 30 min. These cells were then washed and resuspended in fresh medium. Moreover, the influence of environmental temperature, pH, metabolic inhibitors, and potential competitive inhibitors was evaluated. The influence of temperature was examined by comparing antibiotic uptake at 4 and 37°C. The pH profile of ofloxacin uptake in

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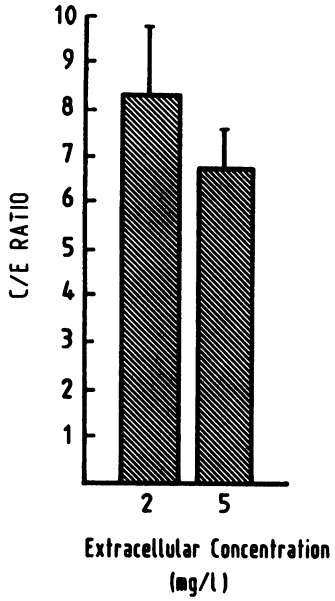

FIG. 1. Uptake by human PMN of ofloxacin at different extracellular concentrations. Incubations were carried out for 20 min.

medium preadjusted to different external pH values (5, 7.2, and 9) by the addition of 10 N HCl or 10 N NaOH was measured. Sodium fluoride (Sigma Chemical Co., St. Louis, Mo.) and sodium cyanide (Sigma) were used as metabolic inhibitors at 1×10^{-3} and 5×10^{-3} M. The PMN in Hanks balanced salt solution with or without metabolic inhibitors were incubated for 30 min at 37°C. Ofloxacin at a final concentration of 5 μg/ml was then added, and the uptake was measured as described above. Other substances were evaluated for possible competitive inhibition of ofloxacin uptake. These materials included nucleosides (adenosine [Sigma]), D-glucose (Sigma), and L-amino acids (glycine, leucine, and lysine monohydrochloride [E. Merck AG, Darmstadt, Federal Republic of Germany]). These substances were added to the cells at the same time as ofloxacin. The uptake of ofloxacin was measured as described above.

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

Controls without antimicrobial agents were always used to determine the background fluorescence. Moreover, since the measurement of ofloxacin is indirect and dependent upon its fluorescence, controls were always used to evaluate the effect of inhibitors and substrates on the fluorescence of ofloxacin in cell-free systems.

Statistical analysis of data. Data were expressed as mean ± standard deviation. A statistical analysis was performed by Student's t test, with $P < 0.05$ considered significant.

RESULTS

Uptake of antimicrobial agents by PMN. Figure 1 shows the C/E ratios of ofloxacin incubated with human PMN for 20 min. With extracellular concentrations of 5 μg/ml, the C/E ratio of ofloxacin was 6.7 ± 1.4. These values were slightly higher when extracellular concentrations of 2 μg/ml were used. Ofloxacin was taken up rapidly. The maximal C/E ratio

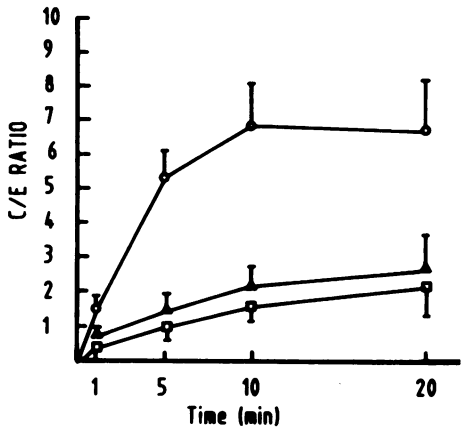

FIG. 2. Influence of cell viability and environmental temperature on ofloxacin uptake by human PMN. Values are mean ± standard deviation of four experiments. Symbols: ○, 37°C, viable cells; △, 37°C, dead cells ($P \leq 0.05$); □, 4°C, viable cells ($P \leq 0.05$).

was obtained after a 10-min incubation (Fig. 2). The temperature and viability dependence of ofloxacin accumulation in PMN are shown in Fig. 2. The uptake of ofloxacin was markedly decreased when viable cells at 4°C or Formalin-killed cells at 37°C were used. Changes in the pH of the medium (pH range 5 to 9) did not affect the uptake of ofloxacin into PMN (data not shown). The effect of metabolic inhibitors on ofloxacin uptake is shown in Fig. 3. Incubation of PMN for 30 min in the presence of different concentrations of sodium fluoride, which inhibits glycolysis, or sodium cyanide, which blocks mitochondrial oxidative mechanism, markedly impaired the penetration of ofloxacin.

Potential competitive substrates of ofloxacin uptake (adnosine, glucose, and L-amino acids) were evaluated. Neither adenosine (Fig. 4) nor different concentrations of glucose affected the uptake of ofloxacin. The presence of the three L-amino acids evaluated, glycine, leucine, and lysine, significantly impaired the uptake rate of ofloxacin by PMN (Fig. 4).

To determine whether ofloxacin that had been taken up by PMN was tightly bound to cellular components, we studied

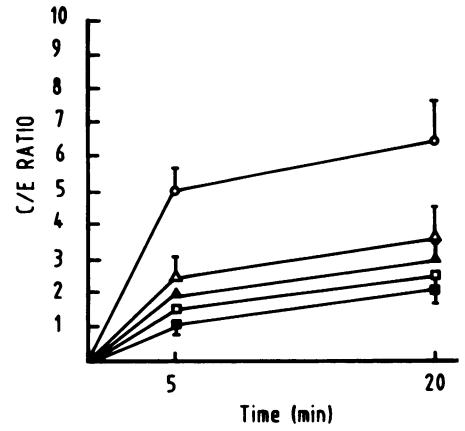

FIG. 3. Influence of inhibitors of cellular metabolism on ofloxacin uptake by human PMN. Values are mean ± standard deviation of four experiments. Symbols: ○, control; △, NaF (1 mM); ▲, NaF (5 mM); □, NaCN (1 mM); ■, NaCN (5 mM). For all but the control, $P \leq 0.05$.

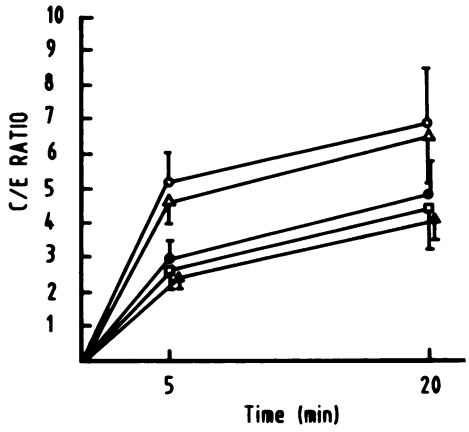

FIG. 4. Uptake of ofloxacin in the presence of 1 mM adenosine, glycine, leucine, and lysine. Values are mean ± standard deviation of four experiments. Symbols: ○, control; △, 1 mM adenosine; □, 1 mM glycine; ▲, 1 mM leucine; ●, 1 mM lysine. For all but the control, $P \leq 0.05$.

the kinetics of efflux (Fig. 5). The reversibility of binding of ofloxacin was rapid, with 65% of the cell-associated drug being lost by 5 min (compare values at 10 and 15 min in Fig. 5).

DISCUSSION

Our results demonstrate that this fluorometric assay is a reliable method for measuring fluoroquinolone uptake by phagocytes. The *C/E* ratios of ofloxacin obtained by this method were similar to those found by others using radioassay (Carlier et al., 27th ICAAC) and HPLC (8). The fluorometric assay is as sensitive as the radiometric method and avoids the use of radiolabeled quinolones. The use of HPLC requires large volumes of phagocytes, very high concentrations of quinolones (50 μg/ml), which are not found in serum at therapeutic concentrations, and several manipulations to prepare the samples, such as deproteinization and ion pair extraction. Although our study involved the use of radioisotopes to measure the PMN volume, this could be avoided if, as Koga (8) has proposed, a fixed estimate of PMN volume of 0.27 pl per cell is accepted.

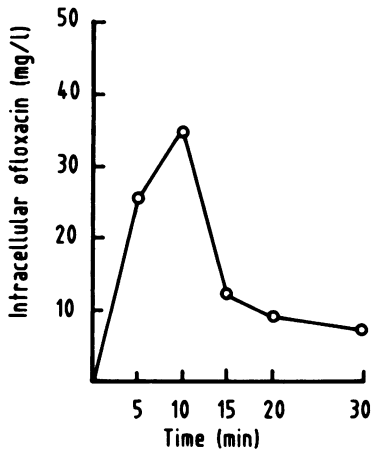

FIG. 5. Efflux of intracellular ofloxacin from human PMN after removal of the extracellular drug. Data are the mean of two experiments at each time point. Cells were centrifuged and resuspended in drug-free medium after 10 min of ofloxacin accumulation.

Using this assay, it has been shown that ofloxacin is concentrated in PMN. This finding supports the clinical and experimental evidence that this antimicrobial agent is effective for the treatment of infections produced by intracellular bacteria (11).

The uptake of ofloxacin by PMN was rapid. Membrane transport of ofloxacin in PMN is an active process, with requirements for cellular viability, elevated environmental temperature, metabolic energy, and establishment of a six- to eightfold cellular-to-extracellular gradient. It is interesting to point out that even an inhibitor of mitochondrial oxidative metabolism, such as sodium cyanide, decreased the uptake of ofloxacin by PMN, since these cells have few mitochondria and do not depend upon oxidative metabolism for energy (6). This phenomenon could be related to secondary or minor activity of this inhibitor in other metabolic functions of phagocytes different from mitochondrial oxidative mechanisms.

Easmon et al. (2, 3), using a bioassay, did not observe any influence of cell viability on ciprofloxacin uptake by phagocytes, and they suggest that a passive rather than an active process is involved. Uptake of ciprofloxacin, ofloxacin, and other quinolones by PMN as measured by HPLC (8) and radiometry (Carlier et al., 27th ICAAC) was, however, markedly dependent on cell viability. The natural substrate for the uptake system by which PMN concentrate ofloxacin was evaluated. Our findings suggest that in contrast to clindamycin, which is transported into human PMN via the nucleoside transport system (6, 8), ofloxacin uptake could be related to an amino acid transport system. Of the substrates evaluated, only glycine, leucine, and lysine impaired the uptake of this antimicrobial agent by human PMN. The chemical or structural characteristics of ofloxacin which allow it to share the amino acid transport system are uncertain, especially if the differences on a charge basis among the amino acids evaluated are considered.

In summary, ofloxacin penetrates into human PMN, reaching high intracellular concentrations by an active mechanism. Although not all antimicrobial agents entering phagocytes will have therapeutic potential, quinolones have been shown to remain active intracellularly against a variety of microorganisms (3, 9, 12). The fluorometric assay to measure intracellular concentrations of fluoroquinolones is less laborious and more specific than the bioassay, is more sensitive than the HPLC assay, and does not require radiolabeled antimicrobial agents.

ACKNOWLEDGMENTS

We thank M. C. Guzman for technical assistance and P. Hidalgo for typing the manuscript.

This work was supported in part by grant PA85-0299 from the Comisión Asesora de Investigación Científica y Técnica, Spain.

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