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Uptake of Fluoroquinolones in Human Monocytes Isolated from Peripheral Blood

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Abstract

The aim of this study was to develop a technique for separating monocytic cells in suspension from peripheral blood to measure the intracellular penetration of three fluoroquinolones (ofloxacin, ciprofloxacin and sparfloxacin).

Mononucleated cells were isolated from the blood on a density gradient with lymphoprep and purified by a specific technique of adhesion and disadhesion on fibronectin. The monocytes were obtained in suspension with 76·8% purity and 97·9% viability. This was a convenient form for measurement of intracellular accumulation by use of the velocity–centrifugation technique. Intra-monocytic penetration of ciprofloxacin, ofloxacin and sparfloxacin was measured at equilibrium after 30-min incubation in the presence of 16 μg mL^{-1} antibiotic. The results revealed low intra-monocytic accumulation of ciprofloxacin (intracellular–extracellular = 1·76) and ofloxacin (intracellular–extracellular = 1·42). The penetration of sparfloxacin was significantly higher (intracellular–extracellular = 2·4).

This study confirms the important differences between human immunocompetent cells in terms of their ability to concentrate quinolones. It also underlines the importance of monocyte–macrophage cellular differentiation as a determinant of antibiotic penetration.

Several pathogenic bacteria, for example, *Listeria*, *Mycobacteria*, *Salmonella* and *Staphylococcus*, are able to survive in human monocytes or macrophages (Fenlon & Cynamon 1986) after ingestion. Persistence of these intracellular pathogens might produce chronic or relapsing infections particularly in immunocompromised patients (Young et al 1992).

Therapy of intracellular infections requires the use of antibiotics which potentialize host cellular defences and have intracellular antimicrobial activity. In this setting the entry of antibiotics into phagocytic cells is essential (Scaglione et al 1993). Fluoroquinolones might be useful for treatment of intracellular infections because their broad spectral activity includes intracellular bacteria and their potential capacity to penetrate and accumulate inside human phagocytic cells (Tulkens 1991).

There have been numerous studies of in-vitro penetration of quinolones into immunocompetent cells but none into monocytes. The results obtained by Desnottes & Diallo (1990) on rabbit alveolar macrophages cannot be extrapolated to human cells. Prokesh & Hand (1982), Koga (1987), Ishiguro et al (1989) and Pascual et al (1991, 1994) have all studied intracellular penetration of quinolones into human polymorphonuclear cells. Rispal et al (1996) have measured the accumulation of quinolones in monocytes but on a continuous cell line (THP1) which has commitment towards macrophage differentiation. Other studies have concerned alveolar macrophages (Hand et al 1984; Carlier et al 1990; Baldwin et al 1992) or peritoneal macrophages (Perea et al 1992) which have important tissular differentiation.

The aim of this work was, first, to examine the experimental conditions necessary for isolation of a suspension of human monocytes from peripheral blood, to validate this cellular isolate and to measure the penetration of three fluoroquinolones into these cells and their accumulation in the cells.

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Materials and Methods

Drugs and chemicals

Reagents and materials used for cell separation and culture were purchased from Polylabo (Strasbourg, France) for lymphoprep, PBS without calcium, magnesium and sodium bicarbonate, ethylene-

diaminetetraacetic acid, gelatin-coated boxes (Corning), and from Sigma (St Quentin-Fallavier, France) for fibronectin (batch 105 H 9304), RPMI 1640, foetal calf serum (batch 627L), myeloperoxidase test, naphthol AS-D chloroacetate esterase test and naphthyl acetate esterase test.

Reagents for density-gradient centrifugation and high-performance liquid chromatography (HPLC) were obtained from Touzart et Matignon (Vitry sur Seine, France) for acetonitrile, Prolabo (Paris, France) for potassium dihydrogen phosphate and perchloric acid (RP Normapur), Merck (Darmstaadt, Germany) for silicon oil (d = 1·06), and Cooper (Melun, France) for paraffin oil (d = 0·875).

CD45 PE and CD 14 FITC monoclonal antibodies for flow cytometry were purchased from Coulter Coultronics (Margency, France).

Fluoroquinolones were kindly supplied by Bayer Pharma Laboratories (Sens, France) for ciprofloxacin (batch 263172), Rhone-Poulenc Laboratories (Antony, France) for sparfloxacin (batch 263172-E) and Diamant-Roussel Laboratories (Romainville, France) for ofloxacin (batch 262).

Monocyte collection by adhesion–disadhesion

The monocytic suspension was obtained from human isogroup peripheral blood, collected in citrated tubes.

Initially mononucleated cells were separated from other blood cells by centrifugation on a density gradient according to a previously described technique (Denholm & Wolber 1991). Blood (10 mL) previously diluted by addition of an equal volume of $Ca^{2+}$and Mg^{2+}-free phosphate-buffered saline (PBS) was layered on the top of lymphoprep (5 mL) in 15 mL vials. Vials were centrifuged (Jouan CR 412) at 600 *g* for 20 min at room temperature and the layer of mononucleated cells was collected by aspiration of the plasma lymphoprep interphase. Collected cell suspension was washed twice with an equal volume of Ca^{2+} and Mg^{2+}-free PBS. The final pellet was obtained by centrifugation at 4°C for 5 min (600 *g*), resuspended in 15 mL RPMI 1640 and this suspension observed by light microscopy to count cells and to check monocytic purity.

Monocytes were separated from other mononucleated cells by specific adhesion with fibronectin, then disadhesion (Freundlich & Avdalovich 1983). Fibronectin-coated Petri dishes were prepared immediately before use. The gelatin-coated dishes were layered with a solution of fibronectin previously prepared by dilution of fibronectin (1 mg) with Ca^{2+} and Mg^{2+}-free PBS (12 mL), and maintained at room temperature for 60 min before the supernatant was discarded by aspiration. To enable cell adhesion, mononucleated cell-suspension (7 mL) was layered on two fibronectin-coated dishes and maintained for 30 min at 37°C in an atmosphere of 95% air–5% CO_2. Unadherent cells were discarded by pipetting supernatant and washing the dish surfaces with Ca^{2+}- and Mg^{2+}-free PBS (3 × 10 mL). Disadhesion of monocytes was achieved by the action of a 10-mM EDTA solution for 15 min at low temperature (0°C) (crushed ice bed). Disadhered cells were resuspended in RPMI 1640 medium to give a final suspension of 4×10^6 monocytes mL^{-1}.

Characterization of the monocytic suspension

Characterization of the monocytic suspension was accomplished by direct microscopic examination, cytochemical studies and immunophenotyping.

Monocytic purity was checked by direct light-microscopic examination of cell morphology on MGG-stained smears. Results are expressed as percent of monocytes, lymphocytes or polymorphonuclear cells. Cellular viability was determined by the trypan blue exclusion method. Cytochemical characterization of cells was performed by evaluation of myeloperoxidase and esterase activity. Myeloperoxidase activity was determined by testing the capacity of cells to secrete hydrogen peroxide (H_2O_2). Benzidine hydrochloride (Sigma) was used according to the method of Kaplow (1965). The staining intensity of esterase granules was evaluated by testing non-specific esterase activity with the method of Yam et al (1971). Cell immunophenotypes were determined by direct immunofluorescence using a flow-cytometer (Cmf Coulter Epics XL) (Jones & Nicholson 1989). Double immunostaining was performed using CD14 FITC-and CD45 PE-marked monoclonal antibodies.

Intramonocytic diffusion of fluoroquinolones

Intramonocytic diffusion of the three fluoroquinolones was measured at equilibrium by incubation of the cellular suspension in RPMI 1640 medium (4×10^6 cells mL^{-1}) at 37°C for 30 min in the presence of the tested antibiotics (16 $\mu g\ mL^{-1}$). After incubation cells were separated from the medium by velocity-gradient centrifugation (Freedman et al 1975) avoiding antibiotic extrusion because of the washing step. Before each experiment micro-vials (Polylabo, 400 μL) for velocity-gradient centrifugation were prepared by successive addition of internal standard solution (sparfloxacin or ciprofloxacin, 6·4 $\mu g\ mL^{-1}$, in 1 M perchloric acid; 80 μL) and an 84 : 16 v/v mixture (d = 1·03) of silicon oil (d = 1·06) and heavy paraffin oil (d = 0·875)

(75 μL). After incubation six samples of cellular suspension (200 μL) were gently layered on the top of the oil mixture. Micro-vials were then centrifuged at 13 000 rev min^{-1} for 5 min (Beckman microfuge 152) to isolate cells from the incubation medium.

Analytical method

Fluoroquinolone concentrations in the extracellular incubation media and perchloric lysates were determined by HPLC with a Kontron serial 400 chromatograph (pumps 420; injector 460; detector UV 430; Kontron, Velizy, France). Chromatographic separation was achieved on a C_{18} bonded reversed-phase polymeric column (Asahipack ODP50) (Prolabo) (15 cm × 4 mm i.d., 5 μm particles) by use of a 15-min linear gradient prepared from two mixtures of pH 3 potassium phosphate buffer (KH_2PO_4, 25 mM) and acetonitrile. The amounts of acetonitrile were 50% and 90% in mobile phases A and B, respectively, and during the run the gradient was changed linearly from 100% A to 70% B. Detection was performed by UV spectrophotometry at 304 nm for sparfloxacin and 280 nm for the other quinolones using 0·005 aufs sensitivity.

Before chromatographic analysis the perchloric intracellular medium lysates were neutralized by addition of 1 M methanolic potassium hydroxide solution to precipitate potassium perchlorate. The supernatant obtained after precipitation was injected (20 μL) directly into the chromatograph. Extracellular incubation media were diluted 1/10 (v/v) with a 1/14 (v/v) solution of perchloric acid containing 6·4 μg mL^{-1} internal standard (sparfloxacin or ciprofloxacin). Before injection these samples were treated in the same way as the cellular lysates.

Standard curves were established daily for each antibiotic. Six standard samples in the concentration range 0 to 2560 ng mL^{-1} were prepared extemporaneously by dilution of a stock solution in perchloric acid. Standards were pretreated, before chromatography, in the same way as the cellular perchloric lysates. Linear regression analysis was used to derive standard curves expressing the relationship between fluoroquinolone concentration and the ratios of peak areas (fluoroquinolone/internal standard). Theses curves were used to determine concentrations of the fluoroquinolones in other samples.

Calculation of intracellular concentration, statistical analysis

Intracellular concentrations of the antibiotics were calculated by dividing the amount of antibiotic present in the intracellular perchloric lysate by the total volume of monocytes calculated according to the formula:

$$V = (4\pi/3 \times R^3)(Y \times 10^6) = Y \times 10^6\ \mu L$$

where $Y \times 10^6$ is the number of monocytes estimated in a 200-μL suspension and R is the mean cellular radius of monocytic cells considered as spheres (6·25 μm). The measured intracellular concentrations were normalized to extracellular concentrations by calculating the intracellular–extracellular concentration ratios. The results are presented as mean ± s.d. Intracellular quinolone penetration levels were compared by analysis of variance then Newman-Keuls tests. $P < 0.01$ was considered as indicative of significance.

Results

Characteristics of the cellular isolate

The isolation technique made it possible to obtain a cellular suspension the monocytic purity of which was estimated from morphology to be 76·8 ± 7·4%. Other cells were mainly lymphocytes; polymorphonuclear leucocytes represented < 0·1% of total cells. According to size-structure, as defined by flow cytometry, the monocytic population represented 74·1% ± 4·1% of the isolated cells, in accordance with morphological characterization. The isolation technique led to a tenfold enrichment of monocytes, with a yield of 38·5 ± 15·2% and a cellular viability always close to 100% (Table 1).

Table 1. Characteristics of the cellular isolates obtained by gradient centrifugation and adhesion–disadhesion. Comparison with previously reported data.

	This study	Denholm & Wolber (1991)	Islam & Wilkinson (1989)
Monocytic purity in the suspension (MGG)	76·8 ± 7·4% (63–84%)	83%	73·8 ± 16·2%
Viability of isolated monocytes (trypan blue)	97·9 ± 1·7%	97%	> 99%
Myeloperoxidase activity	65·4 ± 17·0%		
Esterase activity	74·0 ± 13·0%		
Yield	38·5 ± 15·2%	41%	54·9 ± 5·7%

The values for this study are mean ± s.d., n = 18.

The peroxidase and esterase activities of the isolated cells were evaluated, after staining, by direct morphological examination. Esterase activity was characterized by dark blue granules in the cytoplasm; 74·0 ± 13·0% of the isolated cells contained discrete blue-green granules and were considered as weakly positive. The presence of peroxidase activity in the cells was evidenced by dark-brown staining; 65·4 ± 17·0% of the isolated cells were strongly peroxidase-positive.

Among the monocytes, 64·0 ± 6·8% of the cells expressed CD14 antigen and 65·5% ± 6·9% expressed CD45 antigen. Among cells which contaminated the suspensions, CD14 antigen was not expressed and CD45 antigen was expressed by 25·9% ± 8·0%.

Penetration and accumulation of the three fluoroquinolones in human monocytes

The three fluoroquinolones penetrated the monocytes when cell suspensions were incubated for 30 min in the presence of extracellular concentrations close to 16 $\mu g\ mL^{-1}$. Accumulation of ciprofloxacin and ofloxacin by the monocytes was not significantly different and remained low; the intracellular–extracellular ratio was close to 1·5 at equilibrium (Table 2). Accumulation of sparfloxacin was significantly higher ($P < 0·01$) (Table 2); intracellular concentrations were approximately 2·4-times the extracellular level after 30-min incubation.

Discussion

Measuring the intramonocytic penetration of antibiotics in man requires either the use of continuous cell lines or the development of an isolation procedure from peripheral blood. Continuous cell lines are convenient because they provide unlimited material but their use raises the problem of extrapolation of results to normal cells. We therefore chose to study intracellular penetration of quinolones using monocytes isolated from human blood.

The isolation procedure was developed in this study to furnish a free cell suspension from peripheral blood with optimum monocytic purity and cellular viability, and to obtain a sufficient number of cells ($4 \times 10^6\ mL^{-1}$). Numerous methods for monocytic isolation have been previously described. Elutriative centrifugation according to Figdor et al (1981) leads to very successful monocytic purification with a cellular viability near 100% but this method requires leukapheresis mononucleated cell concentrates to obtain a sufficient yield. Monocytic separation by CD 14-labelled magnetic beads also gives good results and a final suspension 95% pure (Ferrari et al 1993). Considered as non-toxic and biodegradable even if they somewhat affect the viability of cells to which they are bound, they remain non-dissociable. We did not employ this method because we could not estimate the impact of these beads on cellular activation and because of the possible interference of magnetic beads with the centrifugation gradient technique.

Numerous studies (Recalde 1984; Stoll et al 1986) have used a density gradient only to separate monocytes from other blood cells but the monocytic purities of the final suspensions were not good, despite high yields. For this reason techniques have been developed for monocytic separation combining a Ficoll-gradient centrifugation and adhesion on plastic or gelatin surfaces (Islam & Wilkinson 1989). Such techniques were not convenient because our objectives were to separate cells from the incubation medium by centrifugation gradient and the cells remained adherent after they had been isolated. We therefore developed a specific two-step method combining Ficoll-gradient centrifugation and adhesion–disadhesion on a fibronectin matrix and which enabled us to collect cells in suspension. Fibronectin was used because it selectively binds monocytes (Freundlich & Avdalovich 1983). Moreover, the calcium-dependence of monocyte-fibronectin interactions (Yamada 1989) enables us to disadhere cells from the matrix by incubation of adhered cells with 10 mM EDTA solution at low temperature (0°C). This avoids the use of more aggressive methods such as trypsinization or scraping (Jones & Nicholson 1989).

The results obtained in this study demonstrate that this two-step procedure led to isolation of

Table 2. Intracellular penetration and accumulation of ciprofloxacin, ofloxacin and sparfloxacin into isolated monocytes.

	Ciprofloxacin (n = 7)	Ofloxacin (n = 6)	Sparfloxacin (n = 7)
Extracellular concentration tested ($\mu g\ mL^{-1}$)	17·2 ± 2·0	16·4 ± 2·1	16·0 ± 3·6
Intracellular–extracellular concentration ratio	1·75 ± 0·25	1·4 ± 0·4	2·3 ± 0·2*

Each value is the mean ± s.d. of results from six or seven experiments. *$P < 0·01$ significantly different from results for ciprofloxacin and ofloxacin.

Table 3. Intracellular penetration and accumulation of quinolones into immunocompetent cells. Review of the literature.

Cells investigated	Intracellular–extracellular ratio	Reference
Ciprofloxacin		
Human polymorphonuclear cells	6–6·5	Easmon & Crane (1985)
	4·6 ± 0·1	Carlier et al (1987)
	3·49 ± 0·6	Koga (1987)
	5·8 ± 1·6	Pascual et al (1991)
	4	Murdoch & Peterson (1991)
	6	Perea et al (1992)
	3·8 ± 0·9	Garcia et al (1992)
THP 1 continuous cell line	4·29 ± 0·37	Rispal et al (1996)
Mouse peritoneal macrophages	12·7 ± 1·5	Chateau & Caravano (1993)
Human alveolar macrophages	8·1 ± 1·1	Carlier et al (1987)
Ofloxacin		
Human alveolar macrophages	7·1 ± 0·3	Carlier et al (1987)
Human polymorphonuclear cells	2·1 ± 0·1	Carlier et al (1987)
	8·15 ± 2	Koga (1987)
	8·8 ± 2·5	Pascual et al (1991)
	7	Perea et al (1992)
Sparfloxacin		
Human polymorphonuclear cells	4·4 ± 0·3	Carlier et al (1990)
	6.5 ± 1.2	Garcia et al (1992)
THP 1 continuous cell line	9·07 ± 1·07	Rispal et al (1996)

monocytes with purity, viability and yield equivalent to those reported in previous work (Table 1). Notable (but insufficient) monocytic enrichment was obtained at the end of the first centrifugation step—the proportion of monocytes was increased to 33·2 ± 11·8%. The second step enabled the collection of final monocytic suspensions with sufficient purity, 76·8% ± 7·4% (evaluated by morphology), and a viability estimated by the trypan blue exclusion method to be almost 100%.

These final suspensions were used to measure equilibrium intracellular penetration of three fluoroquinolone antibiotics. After 30-min incubation in coated tubes, cells remained suspended without significant adhesion and so separation of cells from the incubation medium was possible by velocity-gradient centrifugation, thus avoiding any loss of intracellular antibiotic (Freedman et al 1975; Koga 1987). Moreover, the number of cells in the final suspensions (4×10^6 cells mL^{-1}) seemed always sufficient for HPLC measurement, with good precision, of intracellular concentrations of the tested quinolones, thus avoiding the use of radiolabelled antibiotics. Our results reveal that the intracellular accumulation of fluoroquinolones in suspension cells was approximately 1·5 for ofloxacin and ciprofloxacin and significantly higher for sparfloxacin (Table 2). Although the monocytic purity of the isolated suspensions (approx. 77%) enables evaluation of antibiotic penetration into monocytes, exact quantification is not possible because of the presence of contaminating lymphocytes. Because of this an error risk must be included in the calculation of intracellular–extracellular ratios.

It is clear from these results that the tested quinolones are poorly accumulated in monocytes and that the intracellular concentrations are lower in monocytes than in other types of cells such as polymorphonuclear cells or macrophages (Table 3). The absence of significant accumulation of the tested quinolones reveals that monocytes cannot be considered as a vector for antibiotic accumulation in tissues.

This study underlines and confirms important differences between immunocompetent cell types with regard to their ability to accumulate antibiotics (Garcia et al 1992; Pascual et al 1994). Moreover, differences exist in the same lineage. In fact the cellular differentiation and activation of monocytic cells appear to be an important determinant of the quinolone accumulation. We suggest that protein kinase C might be the link between cellular differentiation or activation and quinolone uptake. Protein kinase C is an ubiquitous phospholipid-dependent enzyme involved in signal transduction mechanisms associated with cellular growth and differentiation which seems also to be involved in quinolone uptake as suggested by the results of Loo et al (1997).

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