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Uptake of Antibiotics by Human Polymorphonuclear Leukocyte Cytoplasts

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Enucleated human polymorphonuclear leukocytes (PMN cytoplasts), which have no nuclei and only a few granules, retain many of the functions of intact neutrophils. To better define the mechanisms and intracellular sites of antimicrobial agent accumulation in human neutrophils, we studied the antibiotic uptake process in PMN cytoplasts. Entry of eight radiolabeled antibiotics into PMN cytoplasts was determined by means of a velocity gradient centrifugation technique. Uptakes of these antibiotics by cytoplasts were compared with our findings in intact PMN. Penicillin entered both intact PMN and cytoplasts poorly. Metronidazole achieved a concentration in cytoplasts (and PMN) equal to or somewhat less than the extracellular concentration. Chloramphenicol, a lipid-soluble drug, and trimethoprim were concentrated three- to fourfold by cytoplasts. An unusual finding was that trimethroprim, unlike other tested antibiotics, was accumulated by cytoplasts more readily at 25°C than at 37°C. After an initial rapid association with cytoplasts, cell-associated imipenem declined progressively with time. Clindamycin and two macrolide antibiotics (roxithromycin, erythromycin) were concentrated 7- to 14-fold by cytoplasts. This indicates that cytoplasmic granules are not essential for accumulation of these drugs. Adenosine inhibited cytoplast uptake of clindamycin, which enters intact phagocytic cells by the membrane nucleoside transport system. Roxithromycin uptake by cytoplasts was inhibited by phagocytosis, which may reduce the number of cell membrane sites available for the transport of macrolides. These studies have added to our understanding of uptake mechanisms for antibiotics which are highly concentrated in phagocytes.

Interactions between antibiotics and leukocytes may influence the outcome of therapy for bacterial infections. Any effect on the fate of bacteria ingested by phagocytic cells would be especially important (12). Obviously, entry of antibiotics into these cells is required for activity against intraphagocytic organisms. We have previously examined the uptake of 17 antimicrobial agents by polymorphonuclear neutrophilic leukocytes (PMN) and mononuclear phagocytes (6, 7, 10, 11, 13, 17). Human PMN are the crucial phagocytic cells in protection against most bacterial infections. Unfortunately, we found that only a few antibiotics enter these neutrophils efficiently (11, 17). Clindamycin and macrolide antibiotics (erythromycin, roxithromycin) are avidly concentrated in PMN by means of active membrane transport mechanisms (11, 17, 20; W. L. Hand, Antimicrobic Newsl. 5:53–58, 1988). The entry of clindamycin into PMN is via the cell membrane nucleoside transport system (20). Despite considerable effort, we have not identified the specific transport mechanism(s) for macrolide entry into neutrophils (11).

Enucleated human PMN (PMN cytoplasts) contain no nuclei and very few granules but exhibit many characteristics of intact cells (5, 18, 21). Antibiotic entry into these cytoplasts will be independent of concentration within granules. This is important since certain antibiotics (weak bases) have been considered lysosomatropic agents, which accumulate within the granules of leukocytes (3, 14, 15). To further define the mechanisms and intracellular sites of antimicrobial agent accumulation in human neutrophils, we studied the antibiotic uptake process in PMN cytoplasts.

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MATERIALS AND METHODS

Preparation of human PMN. Peripheral venous blood from normal volunteers was collected by venipuncture into heparinized syringes. Granulocytes were isolated by dextran sedimentation and Hypaque-Ficoll density gradient centrifugation (2, 17, 20). When necessary, residual erythrocytes in the granulocyte preparation were lysed with 0.16 M ammonium chloride. Cells were washed and resuspended in tissue culture medium 199 (TC 199) or Hanks balanced salt solution (HBSS) (both from GIBCO Laboratories, Grand Island, N.Y.).

Preparation of PMN cytoplasts. Enucleated human PMN (PMN cytoplasts) were prepared by centrifugation of purified peripheral blood PMN over a discontinuous Ficoll gradient containing cytochalasin B (18). In brief, PMN were suspended in 12.5% (wt/vol) Ficoll solution with 20 μM cytochalasin B (Sigma Chemical Co., St. Louis, Mo.). The cell suspension (1×10^7 to 2×10^7 PMN per ml) was preincubated at 37°C for 5 min. Cells (5 ml) were then layered on a prewarmed, discontinuous density gradient (6 ml of 16% Ficoll on top of 6 ml of 25% Ficoll). Cytochalasin B (20 μM) was present throughout the gradients. Centrifugation was performed for 15 min at 81,000 × *g* in an ultracentrifuge (OTD-50; Ivan Sorvall, Inc., Norwalk, Conn.) with a Sorvall 865B rotor. After centrifugation, a band of cytoplasts was present at the interface of the 12.5 and 16% Ficoll solutions. These enucleated cells were collected with a pipette and washed five times to remove the cytochalasin B.

We confirmed that the cytoplasts contain only a few granules by monitoring the content of two granular enzymes in PMN and cytoplasts. β-Glucuronidase activity, a marker for azurophil granules, was assayed by the release of phenolphthalein from phenolphthalein (β) gluronidase (4). Ly-

sozyme, an enzyme found in both azurophil and specific granules, was quantitated by measuring the rate of lysis of *Micrococcus lysodeikticus* (19). Cytoplasmic lactate dehydrogenase was measured as the increase in A_{340} resulting from the reduction of NAD to form NADH (1).

Determination of antibiotic entry into human PMN and cytoplasts. The uptake of radiolabeled antibiotics by human PMN and PMN cytoplasts was determined by means of a velocity gradient centrifugation technique which we previously described in detail (6–11, 13, 17, 20). Antimicrobial agents evaluated in these experiments were [^{3}H]clindamycin hydrochloride (73.3 mCi/mmol; The Upjohn Co., Kalamazoo, Mich.), [^{14}C]roxithromycin (RU 965) (54.5 mCi/mmol; Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J.), [^{3}H]trimethoprim (60.3 mCi/mmol; Hoffmann-La Roche Inc., Nutley, N.J.), [ring-2-^{14}C]imipenem (15.6 mCi/mmol; Merck Sharp & Dohme, West Point, Pa.), [^{14}C]metronidazole (12 mCi/mmol; Searle Research and Development, Skokie, Ill.), chloramphenicol [dichenoacetyl-1-2-^{14}C] (43.2 mCi/mmol; Dupont, NEN Research Products, Boston, Mass.), [N-$^{14}CH_3$]erythromycin propionate (6.2 mCi/mmol; Eli Lilly & Co., Indianapolis, Ind.), and benzyl[^{14}C]penicillin potassium (55 mCi/mmol; Amersham Corp., Arlington Heights, Ill.).

Human PMN or PMN cytoplasts (10^7 cells per ml) in TC 199 or HBSS were incubated at 37°C with clinically appropriate concentrations (~2.5×10^{-5} M) of radiolabeled antibiotics. Specific antibiotic concentrations were as follows (micrograms per milliliter): clindamycin, 10; roxithromycin, 21; trimethoprim, 7; imipenem, 8; metronidazole, 4.3; chloramphenicol, 10; erythromycin propionate, 20; and penicillin, 10. At predetermined intervals, intact or enucleated PMN with their associated radioactive antibiotic were separated from the extracellular antibiotic by velocity gradient centrifugation in a microcentrifuge tube. This involves centrifugation of PMN or cytoplasts through a water-impermeable barrier of silicone oil into formic acid, which dissolves the cells. The contents of the microcentrifuge tubes were frozen, and the layers were separated by slicing with a razor. The radioactive counts of the lower layer, containing the radiolabeled antibiotic which entered the cells, and the upper layer, containing the antibiotic still in solution, were determined in a liquid scintillation counter. Cellular concentrations of the various agents were calculated after establishing the intracellular volumes (^3H_2O uptake) of PMN and PMN cytoplasts. Antibiotic uptake was then expressed as the ratio of the cellular concentration of antibiotic to the extracellular concentration (C/E).

Characterization of antibiotic uptake in human PMN and PMN cytoplasts. We compared the mechanisms and characteristics of antibiotic uptake in intact PMN and PMN cytoplasts. First, the environmental and metabolic requirements of the uptake process were established for those antibiotics which enter phagocytes and cytoplasts readily (7, 8, 11, 13, 17, 20). Specifically, the influences of cell viability, environmental temperature, and metabolic inhibitors on antibiotic entry into PMN and PMN cytoplasts were determined. The metabolic inhibitors used in this study were sodium cyanide (Sigma) and sodium or potassium fluoride (Mallinckrodt, Inc., St. Louis, Mo.). PMN or cytoplasts were incubated with or without an inhibitor for 30 min at 37°C before determination of antibiotic uptake.

The impact of phagocytosis and other cell membrane stimulation on antibiotic entry into PMN and cytoplasts was determined as we have previously described (9, 11, 20). PMN or PMN cytoplasts (10^7 cells per ml) were incubated

TABLE 1. Uptake of antibiotics by human PMN cytoplasts

Time of incubation (min)	Antibiotic uptake (C/E)[a]							
	Roxithromycin	Clindamycin	Erythromycin propionate	Trimethoprim	Chloramphenicol	Imipenem	Metronidazole	Penicillin G
1	4.61 ± 1.70 (4)	4.96 ± 0.85 (26)	7.65 ± 1.22 (7)		2.29 ± 0.39 (4)	10.72 ± 1.31 (7)		
5	10.11 ± 0.94 (12)	6.89 ± 0.81 (22)	5.58 ± 1.09 (11)	2.40 ± 0.57 (13)		13.14 ± 3.73 (11)	0.49 ± 1.09 (5)	0.00 (4)
15	13.68 ± 1.21 (14)	11.08 ± 0.99 (31)	6.04 ± 1.03 (9)	3.50 ± 0.94 (12)	3.38 (1)	6.37 ± 1.36 (11)	0.77 ± 1.06 (5)	0.83 ± 0.93 (5)
30	13.75 ± 0.97 (14)	9.78 ± 0.88 (35)	5.64 ± 1.41 (11)	2.20 ± 0.54 (13)	3.39 ± 0.31 (4)	5.81 ± 1.20 (11)	1.10 ± 2.10 (5)	0.59 ± 0.93 (6)
60	11.04 ± 1.71 (16)	8.87 ± 1.03 (22)			3.25 (1)	2.56 ± 0.95 (9)	0.55 ± 1.23 (5)	0.26 ± 0.58 (5)

[a] C/E is the ratio of the cellular concentration to the extracellular concentration of antibiotic. Data are means ± standard error of the mean (SEM) of observations at each time period. The number of experiments is shown in parentheses.

TABLE 2. Uptake of antibiotics by human PMN and PMN cytoplasts

Time of incubation (min)	Antibiotic uptake (C/E)[a]							
	Roxithromycin	Clindamycin	Erythromycin propionate	Trimethoprim	Choramphenicol	Imipenem	Metronidazole	Penicillin G
15								
PMN[b]	24.8	9.7	5.4	9.0	2.1	1.0	1.0	0.4
Cytoplasts	13.7	11.1	6.0	3.5	3.4	6.4	0.8	0.8
30								
PMN	28.8	10.5	8.5	8.5	2.4	0.8	1.0	0.3
Cytoplasts	13.8	9.8	5.6	2.2	3.4	5.8	1.1	0.6

[a] Data are means of observations at each time period.
[b] Antibiotic uptake data for PMN are from references 11, 17, and 20 and the present study.

with ingestible microbial particles (opsonized zymosan or *Staphylococcus aureus*) or with a soluble membrane-perturbing agent, concanavalin A (ConA) (Sigma). Cells were preincubated for 30 min at 37°C, washed, and suspended in TC 199, after which uptake of radiolabeled antibiotic was determined.

We have shown that clindamycin is transported into phagocytic cells, including human PMN, by the cell membrane nucleoside system (8, 20). To determine whether this was also true in PMN cytoplasts, we evaluated the ability of adenosine (Sigma) to inhibit clindamycin uptake. The nucleoside was preincubated with PMN cytoplasts for 20 min before determination of radiolabeled antibiotic uptake.

RESULTS

Characteristics of cell population. At least 97% of the granulocytes were neutrophils. Because >90% of these cells were PMN, this cell population is referred to as PMN. Greater than 95% of these phagocytic cells were viable, as judged by trypan blue exclusion.

The recovery of cytoplasts was 88%, compared with the number of PMN in the initial preparation. These PMN cytoplasts contained no nuclei and relatively few granules (β-glucuronidase and lysozyme activity were ~10% of whole-cell values). The mean volumes of 2×10^6 cells (32 experiments) were 0.0009 ml for PMN and 0.00022 ml for cytoplasts. In other words, the cytoplast volume was 24% of the intact cell volume. In contrast, the cytoplasmic lactate dehydrogenase content of the cytoplasts was 39% of that in PMN. This indicates that ~60% of the whole-cell PMN volume is cytoplasm, whereas the nucleus and granules occupy ~40% of the intracellular space.

Antibiotic entry into human PMN and PMN cytoplasts. The uptakes of eight radiolabeled antibiotics by PMN cytoplasts are shown in Table 1. The relative uptakes of these antibiotics in cytoplasts were generally similar to what we have previously demonstrated in PMN (9, 11, 17, 20). For instance, penicillin G penetrated both intact phagocytic cells (9, 17, 20) and cytoplasts poorly. The cellular concentration of this drug was lower than the extracellular level (C/E = 0.6 to 0.8). Experiments with imipenem, a novel β-lactam (carbapenem) antibiotic, provided some unusual findings. This antimicrobial agent rapidly and avidly associated with cytoplasts (C/E = 13 at 5 min), but cell-associated antibiotic declined steadily during the incubation period. This phenomenon was previously observed in studies of imipenem uptake by human PMN and monocytes (10, 11).

Metronidazole achieved a concentration in cytoplasts equal to or somewhat less than the extracellular concentration (C/E = 0.5 to 1.1), which is what we observed in intact PMN (11). Chloramphenicol (a lipid-soluble drug) was concentrated threefold in cytoplasts, much like intact PMN (17). Trimethoprim was concentrated approximately threefold by cytoplasts, and the characteristics of the entry process differed from those of the other drugs we evaluated (see below).

Clindamycin and two macrolide antibiotics (roxithromycin and erythromycin propionate) were markedly concentrated by cytoplasts. The decreased uptake of these antibiotics noted at 60 min was probably due to some deterioration of the cytoplasts with prolonged incubation. Uptake of roxithromycin by PMN cytoplasts (C/E = 14) was greater than that of any other antibiotic, just as it was in intact neutrophils (Table 2) (11).

In Table 2, we compared the uptakes of these eight radiolabeled antibiotics by intact human PMN and cytoplasts. Several points are of interest. Uptake by cytoplasts of those antibiotics (clindamycin, macrolides) which enter by active membrane transport and become highly concentrated in phagocytes was >50 to 100% of that observed in intact PMN (9, 11, 17). Trimethoprim accumulation by intact PMN was greater than that by cytoplasts, although the cellular concentrations were higher at 25°C in both cell preparations (11). Finally, cell-associated imipenem was greater in cytoplasts than in PMN. In both cytoplasts and intact PMN, the cell-associated radiolabel declined with time (11).

Characterization of antibiotic uptake. We established the environmental and metabolic requirements of the uptake process for three antibiotics (clindamycin, roxithromycin, trimethoprim) which achieve high concentrations within cytoplasts. As shown in intact PMN (17, 20), uptake of clindamycin by cytoplasts was dependent on cell viability and a physiologic environmental temperature. There was virtually no binding or uptake of drug by dead (Formalin-treated) cytoplasts or viable cytoplasts at 4°C, and entry was

TABLE 3. Influence of temperature on entry of trimethoprim into human PMN cytoplasts

Time of incubation (min)	Antibiotic uptake (C/E)[a] at:	
	37°C	25°C
5	2.8 ± 1.0 (6)	1.7 ± 0.5 (6), $P = 0.098$
15	4.1 ± 1.9 (6)	6.6 ± 2.8 (6), $P = 0.070$
30	2.6 ± 0.8 (6)	4.5 ± 1.3 (6), $P = 0.017$
60	2.4 ± 1.0 (6)	4.4 ± 1.5 (6), $P = 0.035$

[a] Data are means ± SEM of observations at each time period. The number of experiments is shown in parentheses. *P* values reflect differences between 37 and 25°C groups.

TABLE 4. Effects of membrane-stimulating agents on uptake of roxithromycin by human PMN cytoplasts

Time of incubation (min)	Antibiotic uptake (C/E)[a]			
	Control (TC 199)	Zymosan	*S. aureus*	ConA
1	6.1 ± 1.1 (15)	5.6 ± 1.0 (8)	3.8 ± 0.9 (6)	4.1 ± 1.0 (8)
15	14.5 ± 2.0 (15)	12.5 ± 2.2 (8)	8.3 ± 1.4 (6)	11.1 ± 2.3 (7)
30	19.1 ± 1.5 (15)	13.1 ± 1.4 (7)[b]	10.3 ± 1.8 (6)[b]	12.3 ± 2.4 (8)[b]
60	16.9 ± 1.8 (6)	10.3 ± 2.1 (4)[c]	6.4 ± 1.3 (2)	9.5 ± 1.2 (4)[b]

[a] Data are means ± SEM of observations at each time period. The number of experiments is shown in parentheses.
[b] Significantly different from control (TC 199 only), $P < 0.05$.
[c] Compared with control (TC 199 only), $P = 0.06$.

greater at 37°C than at 25°C. The effect of environmental temperature on trimethoprim accumulation by cytoplasts was quite surprising. There was a greater intracellular concentration of this antibiotic at 25°C than at 37°C (Table 3). This inverse temperature-uptake relationship with trimethoprim also occurs in intact PMN (11) and was not observed with any of the other antibiotics we have studied (11, 17; data not shown).

Next, we determined the effects of metabolic inhibitors on the uptake of clindamycin and roxithromycin by cytoplasts. Neither cyanide, an inhibitor of oxidative metabolism, nor fluoride, which inhibits glycolysis, had any definite effect on entry of clindamycin into cytoplasts. Cyanide had no influence on roxithromycin uptake, but at 30 min fluoride (10^{-3} M) was slightly inhibitory to entry of the drug (C/E of 12.7 versus control of 15.4, $P = 0.04$).

We previously showed that ingestion of microbial particles (but not stimulation with ConA) decreased entry of roxithromycin into PMN (11). In the present study, we found that exposure to microbial particles (*S. aureus*, zymosan) inhibited roxithromycin uptake by cytoplasts as it had in neutrophils (Table 4). Unlike the experiments with PMN, ConA stimulation of cytoplasts also reduced uptake of roxithromycin.

Clindamycin enters intact phagocytic cells by means of the cell membrane nucleoside (adenosine) transport system (8, 20). To determine whether this transport system also accounts for clindamycin uptake in cytoplasts, we attempted to block entry of the antibiotic with adenosine (competitive inhibition). At a concentration of 10^{-3} M, adenosine inhibited clindamycin uptake throughout the incubation period (Table 5).

DISCUSSION

Human PMN cytoplasts, which have no nuclei and only a few granules, maintain many of the cell membrane-associated functions of intact neutrophils (5, 18, 21). Studies of radiolabeled antibiotic uptake in PMN cytoplasts yielded results similar to those we observed in intact cells (9, 11, 17, 20; W. L. Hand, Antimicrobic Newsl. 5:53–58, 1988). Specifically, antibiotics which are highly concentrated in PMN also achieve high levels in cytoplasts. Thus, clindamycin, macrolides (roxithromycin, erythromycin), and trimethoprim entered cytoplasts in a very efficient fashion. Chloramphenicol, a lipid-soluble drug, was concentrated approximately threefold in cytoplasts, just as it is in whole cells. Drugs such as penicillin G and metronidazole do not enter either cytoplasts or intact phagocytic cells well.

An interesting finding was that imipenem rapidly and avidly associated with cytoplasts, followed by a progressive decline in cell-associated drug during the incubation period. This was similar to what we found in studies with intact PMN (11). The basis for this phenomenon is uncertain, but possible explanations can be noted. First, the antibiotic may simply bind to the cell membrane and then dissociate over time. Second, the radiolabeled antibiotic (*N*-formimidoyl thienamycin) may be metabolized or altered by a cellular inactivation mechanism which is not inhibited by the cilastatin component of the drug combination (16).

Other investigators have reported that clindamycin enters the isolated lysosomes of human PMN (14, 15). This is not necessarily reflective of what occurs in intact cells, and we found that almost as much clindamycin enters cytoplasts as intact PMN. Carlier et al. (3) fractionated PMN and macrophages after entry of macrolide antibiotics and found one-third to one-half of the intracellular roxithromycin and erythromycin in the granule fractions. This correlates rather well with our observation that cytoplasts, which have very few granules, accumulated more than one-half as much roxithromycin and two-thirds as much erythromycin as intact cells. Thus, from these studies one would assume that most of the clindamycin or macrolide entering neutrophils is in a location, presumably cytoplasm, other than the granule fraction.

Next, we examined the characteristics of the uptake process for those antibiotics which were highly concentrated in cytoplasts. Trimethoprim accumulation by cytoplasts exhibited the same inverse temperature-uptake properties that we observed in intact PMN (11). The cellular concen-

TABLE 5. Competitive inhibition by adenosine of clindamycin entry into human PMN cytoplasts

Time of incubation (min)	Antibiotic uptake (C/E)[a]		
	Control (HBSS)	Adenosine (1 mM)	Adenosine (0.1 mM)
1	3.8 ± 1.2 (6)	2.0 ± 0.9, $P = 0.015$	4.9 ± 1.0
5	7.0 ± 1.8 (5)	4.7 ± 1.3, $P = 0.083$	7.7 ± 2.5
15	10.2 ± 3.5 (4)	4.2 ± 1.8, $P = 0.010$	9.1 ± 2.5
30	11.4 ± 2.3 (6)	5.9 ± 2.5, $P = 0.068$	12.1 ± 2.1

[a] Data are means ± SEM of observations at each time period. *P* values reflect differences between control (HBSS only) and experimental (1 mM adenosine) groups.

tration of this antibiotic was greater at 25°C than at 37°C, a pattern which we have not seen with any of the other tested drugs. Studies in intact PMN have demonstrated a more rapid efflux of trimethoprim from cells incubated at 37°C compared with 25°C (unpublished observations). This may account for the higher intracellular levels at the lower temperature.

We previously showed that the uptake of clindamycin and macrolide antibiotics by phagocytic cells was by active membrane transport (7, 8, 11, 13, 17, 20; W. L. Hand, Antimicrobic Newsl. 5:53–58, 1988). As is true in intact PMN (17, 20), uptake of clindamycin by cytoplasts was dependent on cell viability and a physiologic environmental temperature. Thus, uptake of clindamycin was greater at 37°C than at 25°C. The effects of metabolic inhibitors on clindamycin and macrolide uptake by cytoplasts were also studied. Neither cyanide, an inhibitor of mitochondrial oxidative metabolism, nor fluoride, which blocks glycolysis, altered clindamycin uptake by cytoplasts under the conditions of this study. However, roxithromycin entry into cytoplasts was slightly inhibited by fluoride.

Clindamycin enters phagocytic cells (PMN and macrophages) by means of the cell membrane nucleoside (adenosine) transport system (8, 20). We performed experiments to determine whether this was also true in cytoplasts. Adenosine inhibited the uptake of clindamycin by cytoplasts, presumably by competitive inhibition at membrane transport receptor sites.

Roxithromycin uptake by human PMN was inhibited by phagocytosis of zymosan and *S. aureus* (11). This is probably the result of a reduction in the number of cell membrane sites available for the binding or transport of roxithromycin. Uptake of roxithromycin by cytoplasts was also inhibited by exposure to microbial particles and ConA, a lectin which might interfere with the binding of macrolides to membrane receptors.

In summary, these studies with PMN cytoplasts have added to our knowledge of antibiotic uptake mechanisms in phagocytes. The mechanisms for uptake of antibiotics which are highly concentrated in phagocytic cells appear to be intact in cytoplasts. This would suggest that accumulation of these drugs proceeds independently of granule function.

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