ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1982, p. 373–380
0066-4804/82/030373-08$02.00/0

Vol. 21, No. 3

Antibiotic Entry into Human Polymorphonuclear Leukocytes

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Received 8 October 1981/Accepted 7 December 1981

Since bacteria which survive within phagocytes may produce serious infection, antibiotics which inactivate these intracellular organisms are needed. To establish those factors which mediate entry of antimicrobial agents into human phagocytes, we studied the uptake of 13 radiolabeled antibiotics by peripheral blood polymorphonuclear leukocytes (PMN). At intervals during a 2-h incubation period, antibiotic uptake by PMN was determined by means of velocity gradient centrifugation, which separates the cell-associated antibiotic from the extracellular antibiotic. Penicillin G and three cephalosporin antibiotics penetrated PMN poorly. The ratio of cellular concentration to extracellular concentration (C/E) of these drugs was <0.01 to 0.5. For gentamicin and isoniazid, the C/E values were approximately 0.8 to 1.0. Chloramphenicol, rifampin, and lincomycin, antibiotics with good lipid solubility, were concentrated twofold (C/E = 2) in PMN. Ethambutol (C/E = 5), clindamycin (C/E = 11), and two erythromycin preparations (C/E = 10 to 13) were markedly concentrated within PMN. Clindamycin uptake was rapid: >70% of the total drug entry occurred within the first minute. Accumulation of clindamycin and erythromycin was an active, energy-requiring process, dependent at least in part upon glycolysis. Clindamycin entered PMN by means of an active membrane transport system which was saturable and had a high binding affinity (K_m = 2 mM) and maximum velocity of uptake (V_{max} = 5 nmol/45 s per 10^6 cells). These observations, together with studies of the biological consequences of intracellular antibiotics, should lead to more effective therapy for infections due to intracellular pathogens.

The ability of antibiotics to enter phagocytic cells may be an important factor affecting therapy for infections caused by organisms which survive intracellularly after ingestion. Examples of such facultative intracellular bacteria include *Mycobacterium tuberculosis, Legionella pneumophila, Brucella* spp., *Listeria monocytogenes*, and some strains of *Salmonella* and *Staphylococcus aureus* (29). However, it is well known that an intraphagocytic organism may not be killed even when incubated with certain antibiotics at a level well above the lethal concentration for that particular bacterium (1, 8, 12, 16, 17, 19, 23–28). One possible explanation for this phenomenon is that these antibiotics penetrate phagocytes poorly (2, 18).

We recently demonstrated that antibiotics vary in their abilities to penetrate rabbit alveolar macrophages (AM). The entry of some drugs is extremely limited, whereas others are avidly concentrated by the phagocyte (13). Since granulocytes are the crucial phagocytic cell in protection against many bacterial infections, we examined the uptake of antibiotics by human polymorphonuclear leukocytes (PMN).

MATERIALS AND METHODS

Collection of human PMN. Approximately 30 ml of venous blood was collected in a heparinized syringe from each normal human volunteer, mixed with Dextran (molecular weight, 200,000 to 300,000; GIBCO Laboratories, Grand Island, N.Y.) in a final concentration of 2%, and allowed to sediment. The leukocyte-rich plasma was collected, and neutrophils were isolated by a modified version of the density gradient centrifugation technique described by Boyum (3–5). Briefly, two gradients were prepared from different proportions of diatrizoate sodium (Hypaque; Winthrop Laboratories, New York) and Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden), thus resulting in separate, carefully adjusted specific gravities. The initial gradient (10 parts 32.8% Hypaque + 24 parts 8% Ficoll; specific gravity, 1.077) separated the lymphocytes, basophils, monocytes, and platelets from the neutrophils, eosinophils, and those erythrocytes remaining after sedimentation. The second gradient (10 parts 50% Hypaque + 20 parts 8% Ficoll; specific gravity, 1.119) separated the neutrophils (and whatever few eosinophils were present) from the erythrocytes. Differential counts and viability assays (trypan blue exclusion) of isolated neutrophils were performed. PMN counts were determined by standard counting methods (7). These cells were washed in

TABLE 1. Uptake (C/E) of antibiotics by human PMN

Time of incubation (min)	Mean (C/E ± SEM)					
	Clindamycin	Erythromycin	Erythromycin propionate	Ethambutol	Rifampin	Chloramphenicol
1	7.54 ± 0.57 (7)[a]		0.995 ± 0.005 (2)			
15	9.68 ± 1.03 (11)	2.44 ± 0.47 (5)	5.42 ± 1.06 (5)	1.43 ± 0.28 (6)	2.21 ± 0.24 (5)	2.09 ± 0.29 (3)
30	10.17 ± 0.54 (26)	6.27 ± 1.14 (8)	8.54 ± 0.81 (13)	2.08 ± 0.20 (8)	2.34 ± 0.13 (5)	2.38 ± 0.46 (3)
60	10.46 ± 0.99 (10)	8.16 ± 1.00 (5)	7.48 ± 1.40 (5)	3.27 ± 0.39 (6)	2.43 ± 0.15 (5)	2.63 ± 0.47 (4)
120	11.08 ± 1.08 (9)	13.32 ± 1.13 (4)	10.42 ± 1.88 (5)	4.83 ± 0.39 (6)	2.33 ± 0.35 (3)	2.23 ± 0.30 (4)

[a] Number in parentheses indicates number of experiments.

tissue culture medium 199 (TC 199; BBL Microbiology Systems, Cockeysville, Md.) and suspended in the same medium at concentrations of 10^7 viable cells per ml for antibiotic uptake studies.

Techniques and determination of antibiotic uptake by PMN. The following radiolabeled antibiotics were used in this study: [^{14}C]rifampin (3.98 mCi/mmol; Ciba-Geigy Corp., Summit, N.J.), [^{3}H]clindamycin hydrochloride (73.3 mCi/mmol; The Upjohn Co., Kalamazoo, Mich.), [^{3}H]lincomycin hydrochloride (1.44 mCi/mmol; Upjohn), chloramphenicol-[dichenoacetyl-1-2-^{14}C] (43.2 mCi/mmol; New England Nuclear Corp., Boston), [^{14}C]cephalexin (2.92 mCi/mmol; Eli Lilly & Co., Indianapolis, Ind.), [^{14}C]cefazolin (3.97 mCi/mmol; Lilly), [^{14}C]cefamandole nafate (3.96 mCi/mmol; Lilly), [*N*-$^{14}CH_3$]erythromycin (5.9 mCi/mmol; Lilly), [*N*-$^{14}CH_3$]erythromycin propionate (6.2 mCi/mmol; Lilly), benzyl [^{14}C]penicillin potassium (55 mCi/mmol; Amersham Corp., Arlington Heights, Ill.), [*G*-^{3}H]isoniazid (isonicotinic acid hydrazide; 1.9 Ci/mmol; Amersham), [*G*-^{3}H]gentamicin sulfate (514 mCi/mmol; Amersham), and [^{14}C]ethambutol dihydrochloride (0.6 mCi/mmol; Lederle Laboratories, Pearl River, N.Y.).

These antibiotics were added to PMN suspensions in concentrations of 2.0×10^{-5} M to 3.1×10^{-5} M (containing 0.03 to 0.09 mCi of radioactivity per ml), which are similar to clinically appropriate serum levels. Concentrations of specific antibiotics were as follows: clindamycin, 2.35×10^{-5} M (10 μg/ml); lincomycin, 2.46×10^{-5} M (10 μg/ml); penicillin, 2.67×10^{-5} M (10 μg/ml); rifampin, 2.44×10^{-5} M (20 μg/ml); chloramphenicol, 3.1×10^{-5} M (10 μg/ml); isoniazid, 2.43×10^{-5} M (3.3 μg/ml); cephalexin, 2.88×10^{-5} M (10 μg/ml); cefazolin, 2.2×10^{-5} M (10 μg/ml); cefamandole, 1.95×10^{-5} M (10 μg/ml); ethambutol, 2.5×10^{-5} M (6.9 μg/ml); erythromycin, 2.5×10^{-5} M (18.4 μg/ml); erythromycin propionate, 2.5×10^{-5} M (19.8 μg/ml); and gentamicin, 2.5×10^{-5} M (18 μg/ml).

The cell-antibiotic mixtures were incubated at 37°C; at intervals, 0.2-ml aliquots of the incubation solution (containing 2×10^6 PMN) were removed, and antibiotic uptake by PMN was measured. Cell counts taken at intervals during incubation were within 10% of the initial count. A velocity gradient technique (13) which employs a miniature centrifuge (Microfuge B; Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) was used for the determination of radiolabeled drug uptake by PMN. Twenty microliters of 88% formic acid was layered on the bottom of each 400-μl microcentrifuge tube and then covered with 150 μl of silicone oil (William F. Nye, Inc., New Bedford, Mass.) The antibiotic-cell suspension (200 μl) was then layered over the oil, and the tubes were centrifuged at $12,000 \times g$ for 3 min, causing the PMN to pass through the water-impermeable oil into the formic acid, which dissolved the cells. Thus, the lower layer contained the radiolabeled antibiotic taken up by the PMN and the upper layer contained the extracellular antibiotic. The contents of the tubes were frozen at −50°C, and the frozen liquid layers were separated with a razor. Each layer was placed in a vial containing toluene, PPO (2,5-diphenyloxazole), and POPOP [1,4-bis-(5-phenyloxazolyl)benzene] and then counted in a Beckman LS-350 liquid scintillation system. Radioactive counts were expressed as disintegrations per minute.

Determination of the intracellular volume was performed by adding ^3H_2O (New England Nuclear) to the PMN suspensions, allowing equilibration, and then, by using the velocity gradient centrifugation technique described above, separating the cells from the extracellular medium. From the values obtained from this procedure, PMN-associated antibiotic concentrations were calculated (from micromoles of drug per intracellular volume of 2×10^6 cells) and expressed as a ratio of the cellular concentration to extracellular concentration (C/E) (13).

Characterization of antibiotic uptake. Further studies were done to elucidate the mechanism of antibiotic uptake by PMN. We determined the influence of cell viability, using PMN killed by exposure to 10% Formalin for 30 min. These cells were then extensively washed and suspended in fresh medium. The role of environmental temperature in determining drug uptake was also evaluated. The following metabolic inhibitors were used at concentrations of 10^{-2} or 10^{-3} M or at

TABLE 1—*Continued*

Lincomycin	Isoniazid	Gentamicin	Cephalexin	Penicillin G	Cefamandole	Cefazolin
0.90 ± 0.50 (2)	1.11 ± 0.005 (2)	1.03 (1)	0.57 (1)	0.40 ± 0.37 (2)	<.01 (2)	<.01 (2)
1.58 ± 1.1 (2)	1.37 ± 0.46 (2)	0.73 (1)	0.55 (1)	0.26 ± 0.11 (2)	<.01 (2)	<.01 (2)
1.70 ± 0.3 (2)	1.50 ± 0.12 (2)	0.83 (1)	0.57 (1)	0.24 ± 0.07 (2)	<.01 (2)	<.01 (2)
1.78 ± 0.02 (2)	1.04 ± 0.04 (2)	0.84 (1)	0.55 (1)	0.16 ± 0.13 (2)	<.01 (2)	<.01 (2)

both concentrations: sodium cyanide (Sigma Chemical Co., St. Louis, Mo.), potassium fluoride (Mallinckrodt, Inc., St. Louis, Mo.), and iodoacetamide (Sigma). Cells in TC 199 with or without inhibitor were incubated for 30 to 60 min at 37°C. The radiolabeled antibiotic was then added, and uptake was measured as described above.

We made a kinetic analysis of clindamycin accumulation in PMN by exposing the cells to a wide range of concentrations of [^{3}H]clindamycin for 45 s. Velocity of transport was determined for each concentration, and a double-reciprocal (Lineweaver-Burk) plot of uptake velocity versus concentration data was constructed. This allowed us to determine the binding affinity (K_m) and maximum rate of uptake (V_{max}).

Efflux of clindamycin and erythromycin propionate was determined after incubating PMN for 30 min in TC 199 containing the radiolabeled antibiotic. The cells were then collected by centrifugation and rapidly suspended in fresh medium without the antibiotic. Intracellular and extracellular concentrations of antibiotics were quantitated at various intervals.

RESULTS

Characteristics of the cell population. Differential counts determined after the cell separation procedure revealed that at least 97% of the isolated leukocytes were neutrophils, with an occasional lymphocyte, eosinophil, and, rarely, a monocyte present. PMN comprised the vast majority (92 to 98%) of the neutrophil population. Thus, this cell population will be referred to as PMN. All PMN were viable, as judged by their ability to exclude trypan blue. This observation agrees with previous findings (5, 9). Furthermore, studies of PMN chemotaxis, a sensitive indicator of cell function, have shown that this isolation technique does not injure PMN (9). Virtually all cells remained viable during the incubation procedures, except as noted below (iodoacetamide exposure).

Antibiotic uptake by PMN. Penicillin G, cefazolin, and cefamandole penetrated PMN poorly. The cellular concentration of these antibiotics remained much lower than the extracellular concentration during the entire 120-min incubation period (C/E = <0.01 to 0.2; Table 1). Cephalexin attained an intracellular concentration that was about 50% that found extracellularly (i.e., C/E = 0.5). Gentamicin and isoniazid achieved somewhat higher intracellular levels (C/E = 0.8 and 1.0, respectively). The more lipid-soluble antibiotics, chloramphenicol, rifampin, and lincomycin, were concentrated approximately twofold in PMN.

In contrast with the other antibiotics tested, ethambutol, erythromycin, erythromycin propionate, and clindamycin were markedly concentrated by PMN (C/E = 5 to 12; Table 1). Clindamycin and the erythromycin drugs attained intracellular concentrations greater than 10 times the extracellular concentration during the incubation period. The entry of clindamycin into PMN was extremely rapid: more than 70% of the total uptake occurred during the first minute. Uptake of clindamycin was essentially complete by 30 min. Uptake rates of ethanbutol and the erythromycin drugs were somewhat slower.

Energy requirements of antibiotic uptake by PMN. Further studies were done to define the uptake rates of rifampin, chloramphenicol, ethambutol, erythromycin propionate, and clindamycin by PMN.

Uptake of erythromycin propionate and clindamycin by PMN requires active transport; this requirement is underscored by the dependency of uptake upon cell viability and physiological environmental temperature. Formalin-killed cells rapidly bound substantial quantities of

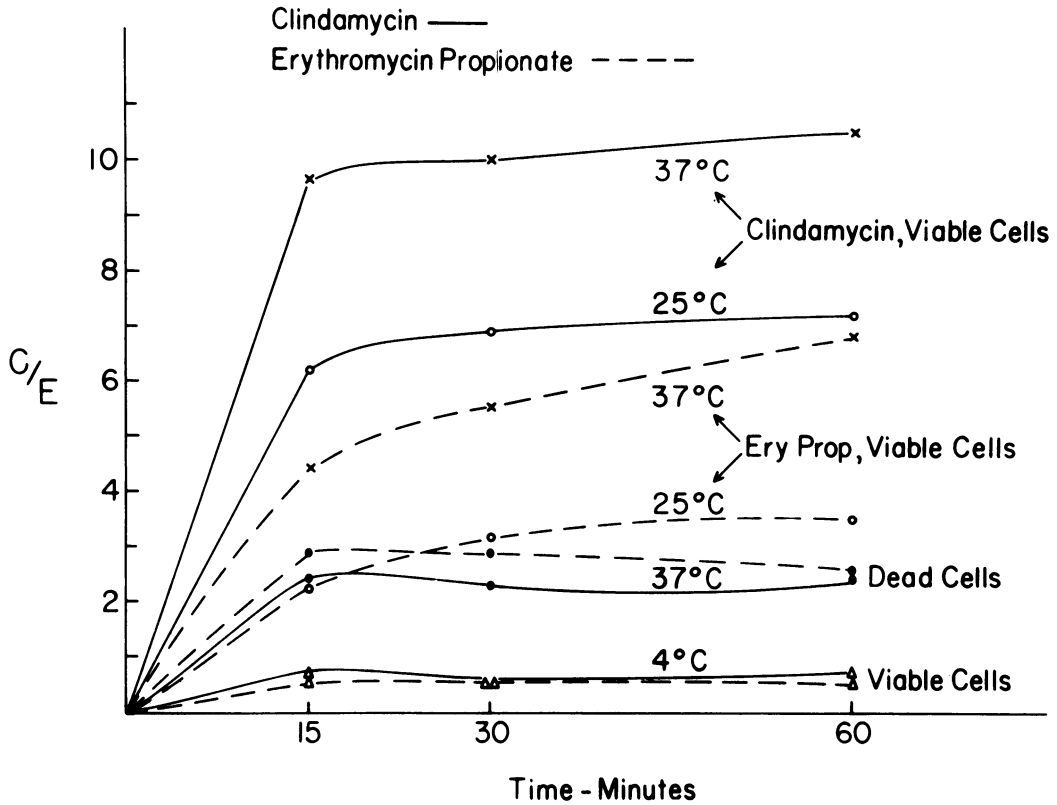

FIG. 1. Dependence of [^{3}H]clindamycin hydrochloride and [N-$^{14}CH_3$]erythromycin propionate entry into human PMN on environmental temperature and cell viability. Dead cells are Formalin-killed PMN. Mean C/E values of two observations at each time period are shown.

these drugs, but no additional uptake occurred with continued incubations. At room temperature (25°C), the accumulation of these drugs by PMN was less than that which occurred at 37°C (Fig. 1; Table 2).

Uptakes of rifampin and chloramphenicol at 37°C were apparently unrelated to PMN viability (Table 2), implying that phagocyte penetration by these lipid-soluble antibiotics is a function of simple solubility partition. The failure of rifampin, a large dipolar ion, to enter viable cells at 4°C may reflect decreased drug solubility in a cell membrane altered by low temperature.

TABLE 2. Influences of cell viability and environmental temperature on antibiotic uptake (C/E) by human PMN

Expt group (incubation temp)[a]	C/E[b]			
	Rifampin	Chloramphenicol	Clindamycin	Erythromycin propionate
Viable cells (37°C)	2.7	3.0	10.5	6.8
Dead cells (37°C)	2.5	2.4	2.5	2.6
Viable cells (25°C)	2.7	3.1	7.2	3.5
Viable cells (4°C)	0.8	2.7	0.6	0.5

[a] Incubations were for 60 min.
[b] Values are means of two experiments.

Ethambutol uptake by dead PMN was most unusual. The maximum C/E was achieved by 15 min and then steadily fell throughout the remainder of the incubation period. Uptake at 4°C was markedly decreased, which, as noted above, may indicate plasma cell membrane alteration by low temperature (Table 3).

We employed various metabolic inhibitors to further elucidate the active clindamycin transport system (Fig. 2). None of the inhibitors had a statistically significant effect on clindamycin uptake after a 1- or 5-min exposure of PMN to the antibiotic. However, at 30 min, potassium fluoride, an inhibitor of glycolysis, significantly reduced clindamycin uptake. Sodium cyanide, which blocks mitochondrial oxidative metabolism, had no effect; one might have anticipated this, since the PMN has few mitochondria and

TABLE 3. Influences of cell viability and environmental temperature on ethambutol uptake (C/E) by human PMN

Time of incubation (min)	C/E[a]			
	Viable cells, 37°C	Dead cells, 37°C	Viable cells, 25°C	Viable cells, 4°C
15	1.4	4.6	0.8	0.5
30	1.9	3.7	0.8	0.6
60	3.0	2.4	0.8	0.5
120	4.9	1.5	1.2	0.5

[a] Values are means of three experiments.

does not depend upon oxidative metabolism for energy. Iodoacetamide, a sulfhydryl group binder, also decreased clindamycin uptake at 30 min, but this was associated with loss of cell viability.

Kinetic analysis of antibiotic uptake by PMN. A kinetic analysis of clindamycin accumulation by PMN during the first 45 s of exposure to the antibiotic is shown in Fig. 4. A double-reciprocal (Lineweaver-Burk) plot of uptake velocity versus concentration data demonstrates that the clindamycin transport system obeys saturation kinetics and is characterized by a high binding affinity (K_m) and a high maximum velocity (V_{max}) or rate of uptake (Fig. 3).

Clindamycin and erythromycin propionate were not tightly bound to cellular components. Efflux experiments revealed that upon removal of the extracellular antibiotic, these drugs rapidly exited the PMN until equilibrium was again reached. In the case of clindamycin, a C/E of approximately 10 was present at equilibrium (Fig. 4).

DISCUSSION

Many microorganisms produce prolonged or recurrent infection because of their ability to survive intracellularly after ingestion by phagocytes (29). Optimal therapy for infections due to facultative intracellular bacteria requires the use of antibiotics that enter the phagocyte and inactivate these organisms. Neutrophilic granulocytes are essential for protection against and recovery from infection due to many bacterial pathogens. Therefore, we examined the uptake of 13 radiolabeled antimicrobial agents by human neutrophils (PMN). A velocity gradient centrifugation technique, which we had previously demonstrated to be efficient and reproducible in determining antibiotic uptake by rabbit

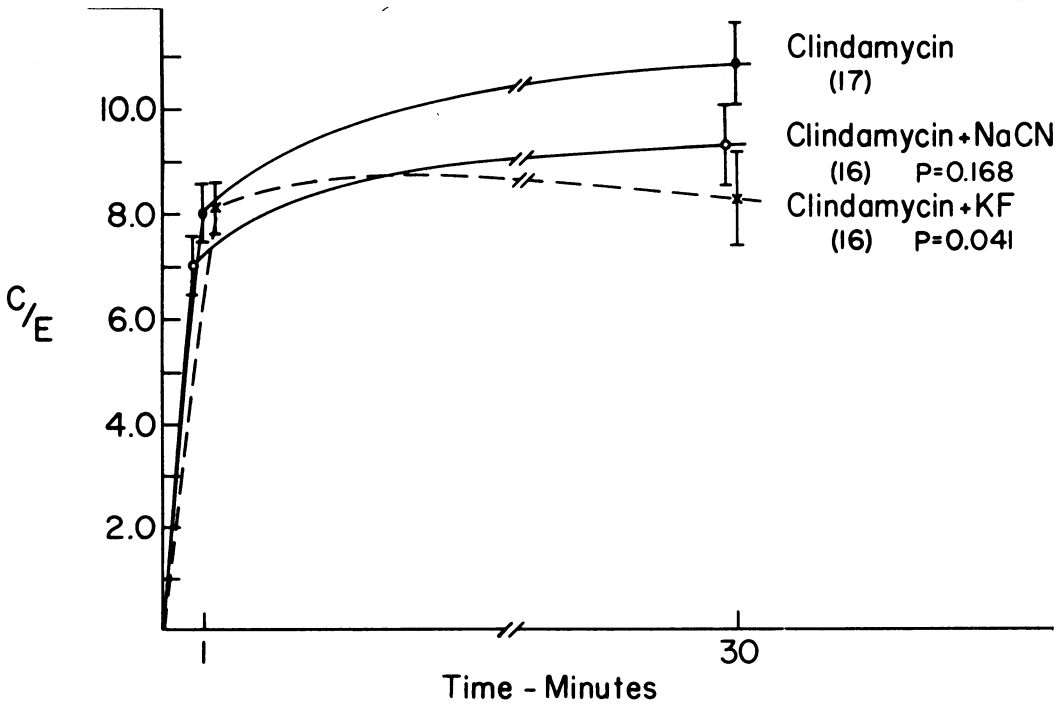

FIG. 2. Effects of metabolic inhibitors on entry of [^{3}H]clindamycin hydrochloride into human PMN. Mean C/E values ± standard errors of the means and number of observations at 30 min (in parentheses) are shown. Comparisons of clindamycin uptake rates of the control groups with those of the experimental groups are denoted by *P* values.

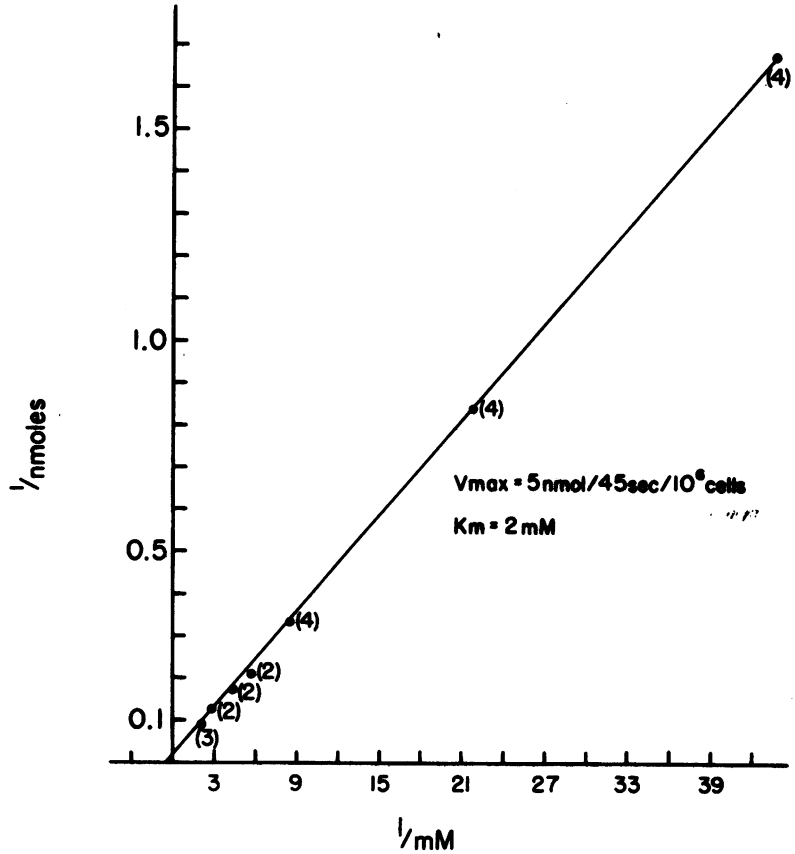

FIG. 3. Kinetic analysis of [^{3}H]clindamycin hydrochloride transport in human PMN (Lineweaver-Burk reciprocal plot of uptake velocity versus concentration data). Cells were incubated for 45 s with a wide range of concentrations of radiolabeled clindamycin, and the transport of the drug at each concentration was determined. Mean uptake and number of observations for each clindamycin concentration (in parentheses) are shown.

AM (13), was utilized in this study. This procedure provides a simple and rapid means of separating the radiolabeled, cell-associated antibiotic from the extracellular drug.

It is of interest to compare the data from our present study of antibiotic entry into human PMN with our previously obtained data on uptake of these drugs by rabbit AM (13). The tested antibiotics varied greatly in their abilities to penetrate phagocytes during the 120-min incubation period. In general, the relative entries of drug groups into the two types of phagocytic cells were similar. Thus, penicillin G and cephalosporin antibiotics failed to penetrate either PMN or AM efficiently. This probably reflects the limited lipid solubility of these drugs and the resulting inability of the drugs to cross biological membranes. However, several previous studies have suggested that antibiotics with good lipid solubility penetrate biological membranes and thus are accumulated by mammalian cells (15, 18, 20). Our observations support this concept, as evidenced by the observation that chloramphenicol, rifampin, and lincomycin, drugs which are lipid soluble, achieved intracellular concentrations (in both PMN and AM) twice that of the extracellular medium. These findings are consistent with the knowledge that one of these lipid-soluble drugs, rifampin, is able to kill intraphagocytic bacteria (10, 16, 18, 19). Uptake of rifampin and chloramphenicol was independent of cell viability, suggesting that entry of these drugs into cells is energy independent and largely due to solubility partition.

Ethambutol was efficiently concentrated by both PMN (C/E = 5) and AM (C/E = 7). The mechanism of ethambutol entry into these phagocytes is uncertain, as evidenced by the observations that metabolic inhibitors failed to influence penetration into either cell type and that cell viability was not required for uptake by PMN.

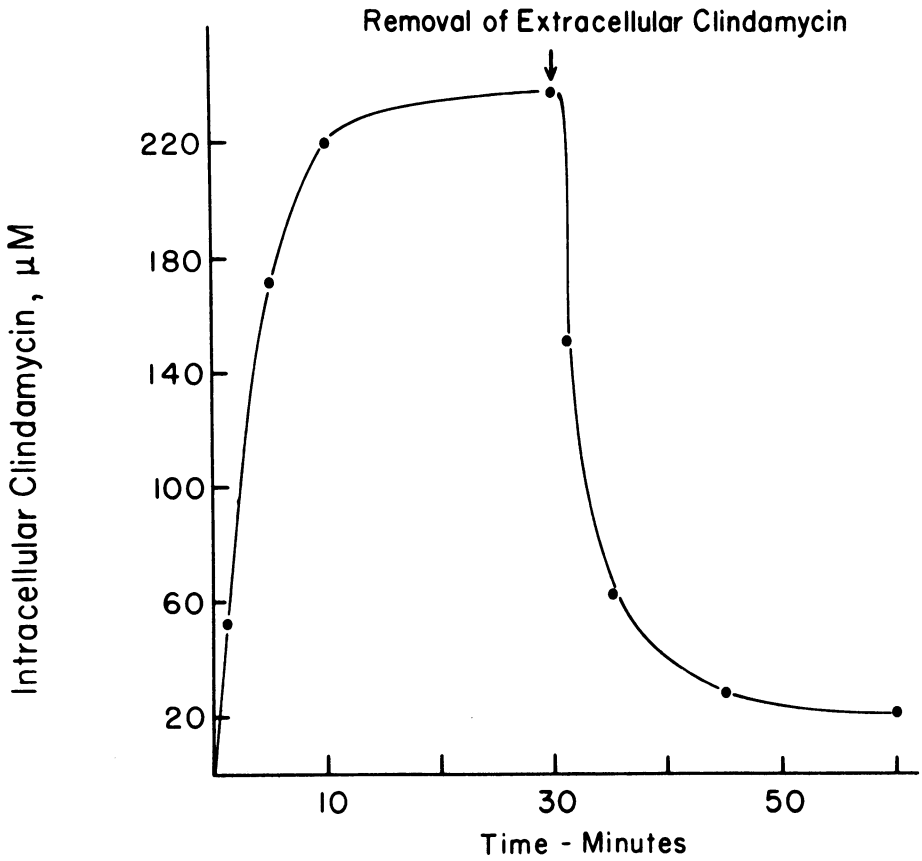

FIG. 4. Efflux of intracellular [^{3}H]clindamycin hydrochloride from human PMN upon removal of the extracellular drug. Clindamycin uptake by PMN for 30 min was followed by the rapid removal of the extracellular drug. Efflux of clindamycin continued until equilibrium (C/E = 10) was again reached.

In contrast with the other antibiotics assayed, clindamycin, erythromycin, and erythromycin propionate were impressively concentrated by PMN and AM. Studies designed to evaluate the antibiotic uptake process revealed that the accumulation of clindamycin and the erythromycins by phagocytes was an active, energy-requiring process. Thus, the uptake of these drugs was characterized by requirements for cellular viability, physiological environmental temperature, metabolic energy, and the establishment of a large cellular-extracellular gradient. Kinetic analysis of active clindamycin transport in PMN and AM revealed that the system was saturable and had a high binding affinity and velocity of uptake. The natural substrate(s) for the uptake system(s) by which PMN concentrate these drugs was not determined. However, we recently demonstrated that clindamycin is transported into rabbit AM via the nucleoside transport system (10a).

Clindamycin and the erythromycins were avidly concentrated by both PMN and AM, but a higher cellular concentration of each drug was attained by AM. This phenomenon is compatible with the knowledge that AM have more complex metabolic machinery and greater adaptive capacity than PMN (6, 11, 21). Our observation that active, energy-requiring transport of antibiotics into PMN is dependent upon glycolysis and that in AM transport is dependent upon mitochondrial oxidative metabolism reflect the major pathways for generation of metabolic energy in each cell type (14, 22).

Efflux of clindamycin and erythromycin from human PMN after removal of the extracellular drug was rapid until the appropriate cellular-extracellular gradient was reestablished (e.g., C/E for clindamycin = 10). These findings indicate that the drugs were not tightly bound to cellular components and suggest that the drugs were not altered by cellular metabolism.

Obviously, the biological consequences of antibiotic interactions with phagocytic cells are important and deserve in-depth evaluation. Clearly, an antibiotic will not inactivate intracel-

lular organisms if the drug fails to enter the phagocyte. However, not all antibiotics entering phagocytes will have therapeutic potential. Both the location and activity of the drug will be of importance in this regard. For example, an antibiotic which enters phagocytic cells might have a beneficial, a deleterious, or a lack of effect upon phagocyte function. Although several drugs inactivate intracellular mycobacteria (17, 27), only rifampin has been shown to kill other intraphagocytic bacteria efficiently (16, 18, 19). Certainly, the consequence of entry into phagocytic cells will require evaluation for each individual antibiotic.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Service of the Veterans Administration. Several pharmaceutical companies kindly provided radiolabeled antibiotics on a complimentary basis; these companies are noted in the text.

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