ANTIBIOTIC BINDING TO HUMAN POLYMORPHONUCLEAR NEUTROPHILS, MOUSE LEUKEMIA L1210 CELLS AND MOUSE THYMOCYTES

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This report describes a system in which antibiotics could be compared for binding to different mammalian cells. These included functional phagocytes (human polymorphonuclear neutrophils; PMNs), non-phagocytic lymphocytes (mouse thymocytes), and non-functional leukocytes (mouse leukemia L1210 cells).

When antibiotics bound to PMNs, they bound about the same to L1210 cells but much less to thymocytes. Combining these data with previous data, the ranking of cells that bound the greatest amount of antibiotics was: PMNs=L1210 cells=blood mononuclear leukocytes> thymocytes>erythrocytes. Thus, antibiotics bind differentially and not indiscriminately to mammalian cells.

Investigations concerning the binding of antibiotics to leukocytes are warranted because binding to leukocytes could influence antibiotic bioavailability, affect antimicrobial effectiveness in abscesses and granulomas, affect important leukocyte host-defense functions, and influence the killing of pathogens sheltered inside leukocytes. Consequently, a variety of systems have been described for studying antibiotic-to-leukocyte interactions. These included measuring the binding of radiolabeled antibiotics[1~5], antibiotic effects on leukocyte functions such as chemotaxis[6~14], antibiotic killing of intraleukocyte microbes[15~23], and the inhibition of [^{3}H]thymidine uptake by staphylococci[24~26] by antibiotics bound to leukocytes[27]. One disadvantage to the latter system was that antibiotics must bind to leukocytes tightly enough to remain bound through two washings by centrifugation. This disadvantage has been circumvented in the current experiments by centrifugation of the antibiotic/leukocyte complex through silicone oil[28].

Using this modified system, it was possible to compare the binding of a variety of antibiotics to functional phagocytes (human polymorphonuclear leukocytes) with non-phagocytic mouse L1210 leukemia cells and mouse thymocytes.

Materials and Methods

Media

Hanks balanced salt solution (HHG) and medium 199 (MHG; Gibco Laboratories, Grand Island, N.Y.) were buffered to pH 7.2 with HEPES (Sigma), 5 mg/ml and contained 0.1% gelatin.

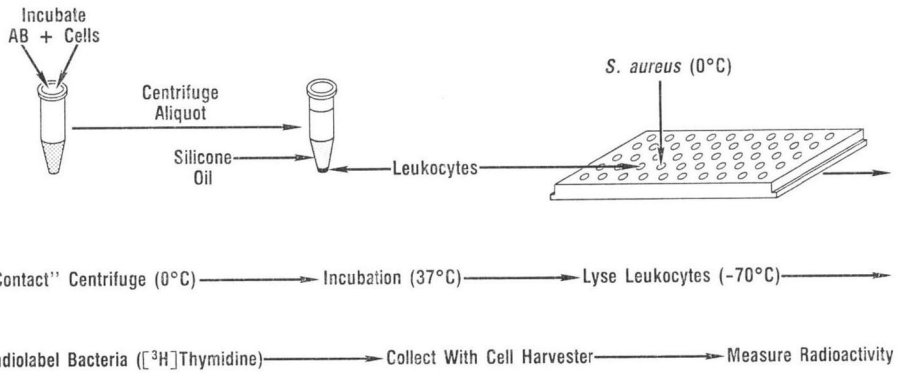
Bacteria

Staphylococcus aureus 502a (UC 9116) was kindly donated by G. L. MANDELL and grown in Tryptone - yeast - glucose broth as described previously[27]. The frozen bacteria were thawed, sonicated, and diluted to 1×10^7 cfu/ml in MHG.

Antibiotics

Antibiotics were obtained from Sigma (St. Louis, MO) with the exception of clindamycin (The

Fig. 1. Outline of antibiotic-to-leukocyte binding assay.



Upjohn Company, Kalamazoo, MI) and rifampin (Calbiochem, La Jolla, CA).

Leukocytes

Human polymorphonuclear neutrophils (PMNs) were isolated as described previously[11]. Thymocytes were obtained from 10- to 15-day old CF-1 mice sacrificed by cervical dislocation after ether anesthesia. The tissue was immersed in ice-cold HHG, pressed through a wire screen, then 2 layers of silk. The thymocytes were washed twice and resuspended in HHG. Mouse leukemia L1210 cells were kindly provided by T. WALLACE, Cancer Research, The Upjohn Company. Frozen L1210 cells were cultured in RPMI 1634 containing 5% fetal calf serum (Hyclone Laboratories, Logan, UT) at 37°C for 24 hours. They were placed into fresh media, 5×10^4/ml, and incubated for an additional 24 hours. The L1210 cells were washed twice and resuspended in HHG to a concentration of 5×10^6/ml.

Antibiotic-to-leukocyte Binding System

An outline of the system is shown in Fig. 1 and has been described in detail elsewhere[27]. After incubation of antibiotic with leukocytes, rather than using centrifugation to wash off extracellular antibiotics, leukocytes were centrifuged through silicone oil (#550, Dow Corning Corp., Midland, MI) leaving antibiotics behind in the aqueous phase[28]. Contact between *S. aureus* 502a and the phagocyte-antibiotic complex was assured by centrifugation at 4°C in 96-well tissue culture trays, phagocytosis was induced by incubation at 37°C, leukocytes were lyzed by freezing and thawing the trays, and assessment of viable bacteria was accomplished by collecting [^{3}H]thymidine labeled bacteria using a cell harvester. An additional modification from the original procedure involved preincubating the frozen bacteria for 1 hour at 37°C before addition of [^{3}H]thymidine; this resulted in increased KCPM. Each value reported represents the mean ±1 standard deviation (using N-1) of quadruplicate samples of each antibiotic concentration.

Quantitation of Antibiotic Bound to Leukocytes

In order to estimate how much antibiotic actually bound to leukocytes, antibiotics were added directly to PMNs and *S. aureus* at 0°C, which effectively blocked binding[5,27]. These trays were then processed in the same manner as those used above in the binding system. The resulting curves of inhibition, which represented the sum contribution of the PMNs and antibiotic, were then superimposed over inhibition curves obtained from the binding system and, in this way, the amount of antibiotic carried by the leukocyte could be estimated.

Results

Comparison of Antibiotic Binding to PMNs, L1210 Cells and Thymocytes

Preliminary experiments with each cell type were performed to estimate the antibiotic concentra-

Fig. 2. Clindamycin binding to PMNs, L1210 cells and thymocytes.

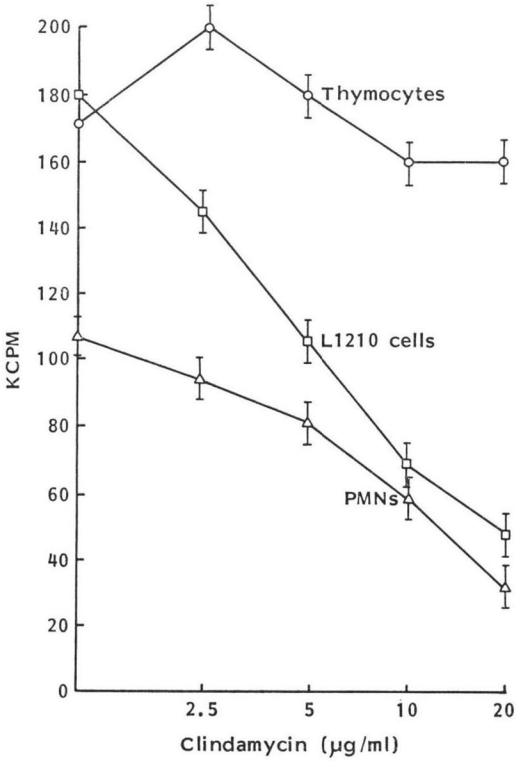
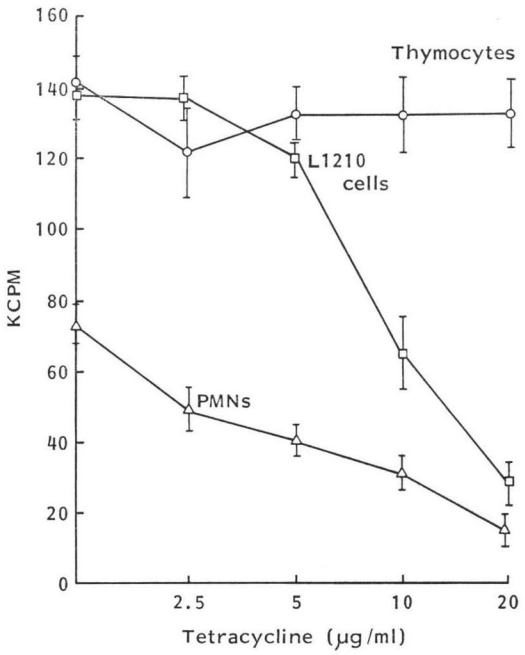


Fig. 3. Tetracycline binding to PMNs, L1210 cells and thymocytes.



tions that should be employed. The results of the experiments with PMNs were in accord with previous results[27]. Moreover, antibiotic binding to L1210 cells appeared to be about the same as PMNs although binding was substantially less to thymocytes. In order to make direct comparisons, the three cell types were then examined simultaneously in the same experiments for antibiotic binding (only those antibiotics that bound to PMNs were tested with the other cell types).

Clindamycin bound to PMNs and L1210 cells in a concentration-dependent fashion (Fig. 2); greater inhibition of [^{3}H]thymidine uptake accompanied increased clindamycin concentrations. However, no binding to thymocytes was observed at concentrations up to 20 μg clindamycin per ml. Tetracycline (Fig. 3) resembled clindamycin in that a concentration-dependent effect was seen with PMNs and L1210 cells, although tetracycline bound somewhat less to L1210 cells. Again, no binding to thymocytes was observed at the highest tetracycline concentration tested (20 μg/ml). Similar results were obtained with novobiocin (Fig. 4) except that inhibitory activity was seen at much lower incubation concentrations compared with clindamycin and tetracycline. In addition, binding to thymocytes was observed. Likewise, fusidic acid (Fig. 5), doxycycline (Fig. 6) and rifampin (Fig. 7) bound to all three cell types in a concentration-dependent manner. Rifampin and fusidic acid inhibited [^{3}H]-thymidine uptake at the lowest incubation concentrations compared with the other antibiotics. Complete inhibition was observed when these antibiotics were incubated with PMNs at concentrations of 0.3 and 0.6 μg/ml, respectively.

Quantitation of Antibiotic Bound to Leukocytes

The differences in the results between antibiotics could suggest that some antibiotics bound to

Fig. 4. Novobiocin binding to PMNs, L1210 cells and thymocytes.

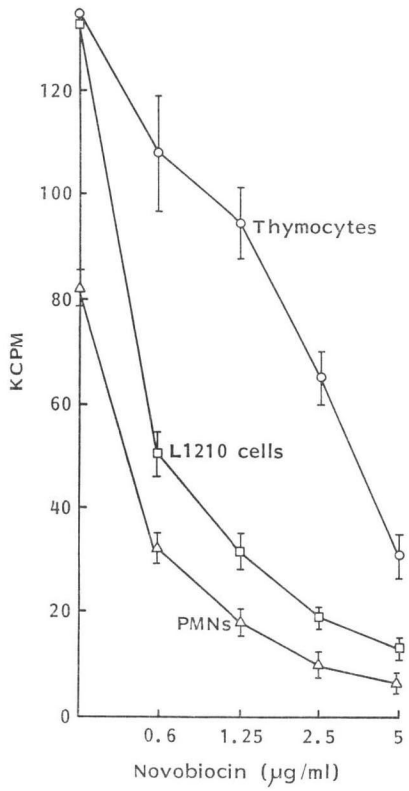
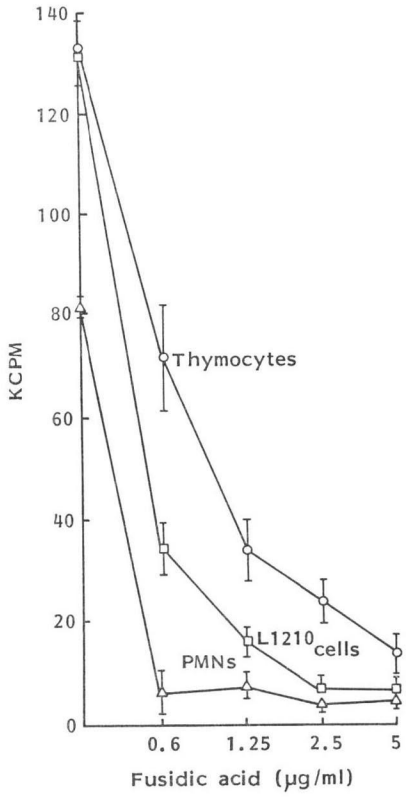


Fig. 5. Fusidic acid binding to PMNs, L1210 cells and thymocytes.



leukocytes more than others. Another possibility was that the same amount bound but the antibiotics differed in terms of their intrinsic potency in inhibiting [^{3}H]thymidine uptake. To examine this latter possibility, a comparison was made of effects on [^{3}H]thymidine uptake when antibiotics were added directly to PMNs and bacteria *versus* effects by the antibiotics after being bound and carried by PMNs. The inhibitory curves generated by direct antibiotic addition were then superimposed over those obtained by antibiotic/leukocyte incubation. In this way, the amount carried by the leukocyte could be estimated.

Representative experiments with clindamycin (Fig. 8), fusidic acid (Fig. 9), and rifampin (Fig. 10) were selected for presentation because of their range of potency in inhibiting [^{3}H]thymidine uptake; clindamycin (weak), fusidic acid (moderate), and rifampin (strong). When clindamycin was incubated at 10 μg/ml, this was equivalent to the direct addition of 50 ng/ml, Leukocyte incubation with fusidic acid at 1.25 μg/ml was equivalent to adding 20 ng/ml; incubation with rifampin at 0.3 μg/ml was equivalent to adding 1 ng/ml. Thus, the differences between the antibiotics was a reflection of their intrinsic inhibitory potencies on [^{3}H]thymidine uptake in addition to their binding capabilities.

The theoretical amount of antibiotic bound was arbitrarily calculated at the concentration at which [^{3}H]thymidine uptake was inhibited by 50% (IC_{50}). This was, for clindamycin, 40 fg/PMN (from Fig. 8, IC_{50}=100 fg/ml divided by 2.5×10^6 PMNs/ml); fusidic acid=8 fg/PMN (Fig. 9, IC_{50}=20 ng/ml); rifampin=0.4 fg/PMN (Fig. 10, IC_{50}=1 ng/ml). Replicate experiments yielded results

Fig. 6. Doxycycline binding to PMNs, L1210 cells and thymocytes.

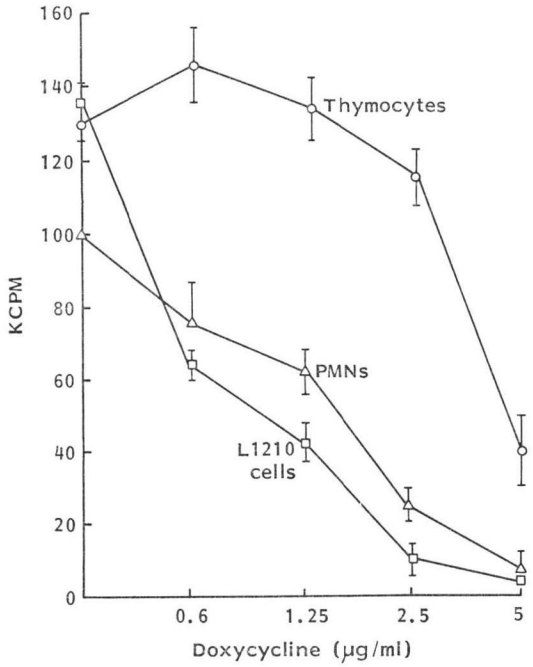
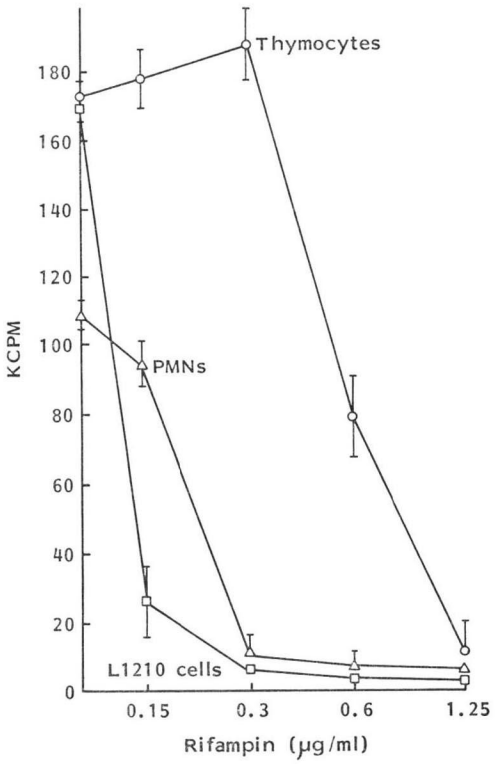


Fig. 7. Rifampin binding to PMNs, L1210 cells and thymocytes.



within 2-fold of the numbers reported. In other experiments, the values calculated were, for tetracycline, 24 fg/PMN; doxycycline=4 fg/PMN; novobiocin=1 fg/PMN.

Discussion

The results of this study lead to the following conclusions. The modified antibiotic-to-leukocyte binding system can be used with a variety of cells including either phagocytic or non-phagocytic leukocytes. Second, antibiotics that bound to phagocytic leukocytes (PMNs) usually bound equally well to mouse leukemia cells, but much less to mouse thymocytes. These results, coupled together with previous work using mononuclear leukocytes and erythrocytes, show that antibiotics bind to different degrees depending on the type of mammalian cell.

PMNs alone caused a reduction in KCPM compared with either L1210 cells or thymocytes (neither of which affected [^{3}H]thymidine uptake by *S. aureus*). The largest reduction was obtained in the experiment shown in Fig. 2 (105 *versus* 180 KCPM); the smallest can be seen in Fig. 6 (100 *versus* 135 KCPM). Thus, this reduction accounted for the discrepancy, for instance, between clindamycin incubated with PMNs *versus* L1210 cells (Fig. 2), and indicates that the discrepancy was due to intrinsic differences between PMNs and L1210 cells in killing *S. aureus*. The lower KCPM seen with clindamycin and PMNs represents an additive effect therefore, whereas the reduced KCPM seen with clindamycin and L1210 cells represents the singular contribution of clindamycin. Similar logic applies to the other antibiotics to explain why lower KCPM were obtained when antibiotics were incubated with PMNs.

A comparison of this antibiotic-to-leukocyte binding system with other types of antibiotic binding systems was made previously[27]: The use of radiolabeled antibiotics is limited by the small number of radiolabeled antibiotics that are available; measuring antibiotic killing of intraleukocyte microbes is

Fig. 8. Effects on [^{3}H]thymidine uptake into *S. aureus*: comparison of clindamycin carried by PMNs with clindamycin added directly to PMNs and *S. aureus*.

Clindamycin was incubated with PMNs followed by removal of extracellular antibiotic using centrifugation through silicone oil at the concentrations shown (○). Clindamycin was also added directly to wells in microculture trays containing *S. aureus* and PMNs at 0°C at the concentrations shown (△). The rest of the binding system was used as described earlier.

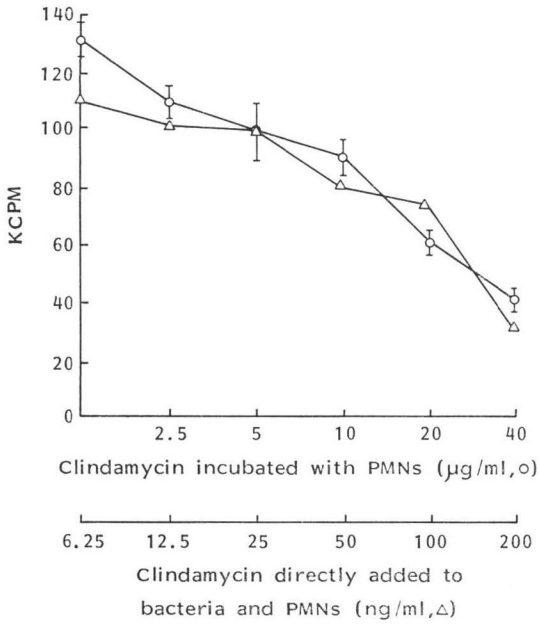
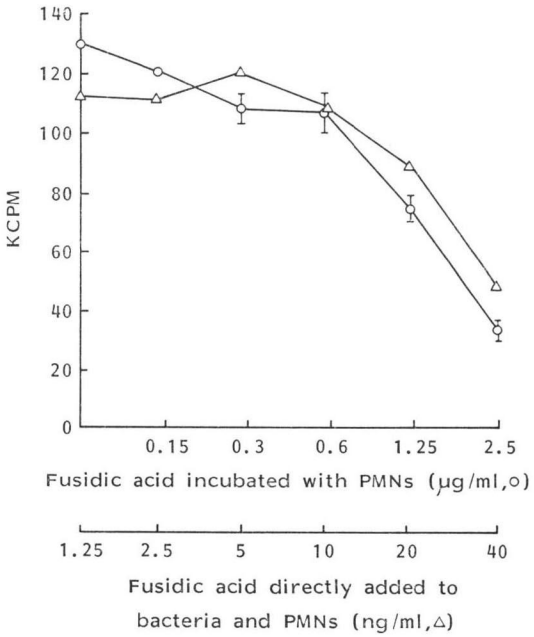


Fig. 9. Effects on [^{3}H]thymidine uptake into *S. aureus*: comparison of fusidic acid carried by PMNs with fusidic acid added directly to PMNs and *S. aureus*.

The experiment was conducted as described over Fig. 8.



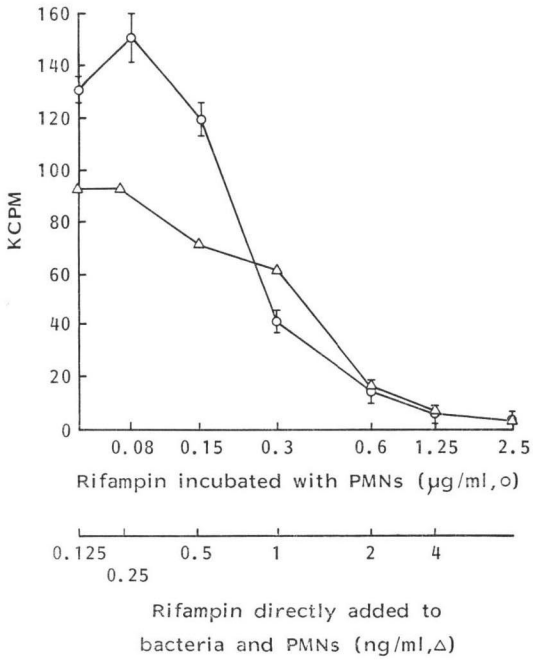
limited because only a few antibiotics possess intraleukocyte killing activity; measuring effects on leukocyte function is difficult because only a few antibiotics affect function. Although our earlier described system was claimed to be fast and facile and not have the disadvantages noted above, one disadvantage was that the antibiotic had to bind tightly enough to remain attached to leukocytes through two centrifugation washings. This problem has now been circumvented by centrifuging the antibiotic/leukocyte complex through silicon oil[28] which leaves the extracellular antibiotic in the aqueous phase. This was an important modification because some antibiotics, *e.g.*, clindamycin, rapidly exit PMNs in the absence of extracellular antibiotic[5].

In the previous study, little difference was observed between PMNs and mononuclear leukocytes in binding clindamycin, fusidic acid, novobiocin, and rifampin; that is, these antibiotics bound about the same to PMNs, and a mixture of blood monocytes and lymphocytes (Table 5, ref 27). In the current study, the following antibiotics bound about the same to PMNs and mouse leukemia L1210 cells; clindamycin, fusidic acid, novobiocin, rifampin, and doxycycline. (A statement of exact equalness is not possible because PMNs alone had the ability to inhibit [^{3}H]thymidine uptake by staphylococci while L1210 cells had no effect.) Tetracycline was the only apparent exception; it bound less to L1210 cells compared with PMNs. All antibiotics bound much less to mouse thymocytes. Taken together, these and previous data suggest that the binding of the antibiotics studied is approximately the same to phagocytic cells (human PMNs), non-phagocytic cells (mouse leukemia L1210 cells), and a mixture of both (human mononuclear leukocytes), but much less to thymocytes and essentially none to human erythrocytes.

The composite results show that antibiotics do not bind to all cell types indiscriminately; antibiotics

Fig. 10. Effects on [^{3}H]thymidine uptake into *S. aureus*: comparison of rifampin carried by PMNs with rifampin added directly to PMNs and *S. aureus*.

The experiment was conducted as described over Fig. 8.



do display specificity in their interaction with mammalian cells. The most obvious differences between the cells are differences in their plasma membranes, cytoplasm size, and presence of granules and nuclei. Although elucidation of the mechanisms involved in antibiotic binding were not addressed in this report, previous studies suggest that the system can be used for that purpose[27], especially with antibiotics that appear to use specific transport mechanisms such as clindamycin[3~5,29]. Moreover, since binding mechanisms can be investigated using cells that differ markedly in both morphology and function, theoretically, this system could be used with any cell that can be disassociated from its native tissue.

The amount of antibiotic bound to PMNs was estimated in the following way. Antibiotics were added at known concentrations directly to PMNs and *S. aureus* 502a at 0°C; this effectively blocked binding. The resulting curves of [^{3}H]-thymidine uptake inhibition, which represented the sum contribution of both PMNs and antibiotic, were then superimposed over curves obtained after the usual incubation. The concentrations at which the KCPM were decreased by 50% were chosen arbitrarily to calculate the amount carried by the PMN. For instance, incubation of 10 μg clindamycin per ml with 2.5×10^6 PMNs/ml was equivalent to the direct addition of 50 ng clindamycin per ml. After making corrections for aliquots and dilutions, this calculated to about 40 fg clindamycin per PMN. Similar treatment of the data lead to the following estimates of antibiotic bound per PMN: Fusidic acid, 8 fg/PMN; rifampin, 1 fg/PMN; novobiocin, 1 fg/PMN; tetracycline, 24 fg/PMN; doxycycline, 4 fg/PMN. Because these calculations were made arbitrarily, at concentrations that inhibited [^{3}H]thymidine uptake by 50%, it is important to re-emphasize that the amount of antibiotic bound could be increased by simply using higher antibiotic concentrations during incubation with leukocytes. Nevertheless, the data may reflect *in vivo* antibiotic cellular distribution because the concentrations employed are close to those obtained in blood during therapy[30].

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