A SIMPLE SYSTEM FOR STUDYING ANTIBIOTIC BINDING TO LEUKOCYTES†

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Studies on the interaction between antibiotics and polymorphonuclear leukocytes (PMLs) usually require the availability of radiolabeled antibiotic, that the antibiotic kill intraleukocyte pathogens, or that the antibiotic affect some function of the leukocyte. The system described here does not have the requirements above but depends instead upon anti-staphylococci activity.

The key features of the system are that, following incubation, extracellular antibiotic was removed from PMLs by centrifugation, contact between *Staphylococcus aureus* 502A and the phagocyte-antibiotic complex was assured by centrifugation at 4°C in 96 well tissue culture dishes, phagocytosis was induced by incubation at 37°C, and assessment of surviving bacteria was accomplished by collecting [^{3}H]thymidine labeled bacteria using a cell harvester.

Antibiotics that were active in this system included naphthalenic ansamycins (rifamycins and streptovaricins), lincosaminides (clindamycins and pirlimycins), coumarins (novobiocin), erythromycin, tetracycline, tyrocidine and paulomycin.

Attempts to understand the interactions between antibiotics and leukocytes generally utilize one of three techniques. The interaction can be studied by measuring the binding of radiolabeled antibiotics[1~5], by measuring antibiotic killing of intraleukocyte microbes[6~14], or by measuring antibiotic effects on some leukocyte function such as chemotaxis[15~28]. These techniques have generated much useful information but each has some disadvantage; for instance, radiolabeled antibiotics are not always readily available (such as those under development), only a few antibiotics are capable of killing microbes inside leukocytes, and not all antibiotics affect leukocyte functions.

The system described here to assess antibiotic-leukocyte interactions demands that the antibiotic possess activity against the test organism (*Staphylococcus aureus* 502A) but does not have the requirements discussed above. The system is facile and fast and was used to confirm interactions discovered using the techniques described above as well as to discover other antibiotics that bind to leukocytes.

Materials and Methods

Chemicals

Rifampicin and rifamycin SV were obtained from Calbiochem (LaJolla, CA); Novobiocin and fusidic acid from Sigma (St. Louis, MO); Benzylpenicillin, gentamicin, tetracycline, erythromycin, vancomycin, chloramphenicol, and kanamycin were obtained by dissolving lyophilized antibiotic present in the wells of Sensititre trays (Gibco Laboratories, Lawrence, MA); Lincosaminide analogs were generously provided by R. D. BIRKENMEYER and B. BANNISTER, coumarins by O. SEBEK, and paulomycins from A. ARGOUDELIS (The Upjohn Company, Kalamazoo, MI). The streptovaricins, rifamycins, and tolypomycin Y were kindly donated by K. RINEHART, University of Illinois, Champaign, IL.

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Purification of Polymorphonuclear Leukocytes (PMLs) and Mononuclear Leukocytes (MNLs)

PMLs and MNLs were prepared using citrated blood, dextran sedimentation, Hypaque-Ficoll centrifigation, and erythrocyte lysis. The leukocytes were suspended in HANK's balanced salt solution without Ca^{2+} or Mg^{2+}. This solution was buffered to pH 7.2 with HEPES, 5 mg/ml, and designated "H/H." The concentration of the stock solution was 5×10^6 leukocytes per ml unless otherwise noted. After "uptake incubation" (see below), the leukocytes were resuspended in 0.5 ml Medium 199 buffered to pH 7.2 with HEPES, 5 mg/ml. This solution was designated "M/H".

Preparation of Erythrocytes

The bottom one-third of the blood remaining after dextran sedimentation was twice-washed by centrifugation at $250\times g$ for 10 minutes with H/H. The erythrocytes were diluted to 5×10^6 erythrocytes per ml. This preparation contained less than 1×10^3 PMLs per ml.

Staphylococcus aureus 502A

S. aureus 502A (UC 9116), kindly donated by G. L. MANDELL, was grown in Tryptone - yeast - glucose broth[14] to stationary phase. The bacteria were washed with M/H by centrifugation and stored over liquid nitrogen at a concentration of 2×10^9 cfu per ml M/H. On the day of the experiment, this suspension was thawed and sonicated for 5 seconds at setting 1 (Branson Instruments, Danbury, CT). Sonication was necessary to obtain individual bacteria from the clumps present; no loss in bacterial viability was observed. This suspension was diluted 1: 10 in M/H, and 50 μl added to each well of a tissue culture dish to yield a final concentration of 1×10^7 cfu per well.

Antibiotic Binding

Antibiotics, 250 μl, at the concentrations noted in the text, were incubated with 250 μl of 5×10^6 blood cells per ml. The incubation was conducted for 20 minutes in 1.5 ml conical polypropylene tubes (Sarstedt Inc., Princeton, NJ) at 37°C. Only 6 tubes were processed at one time in order to minimize the time available for antibiotics to escape from the cells into the wash fluid. The tubes were centrifuged for 15 seconds in a Beckman microfuge (approximately $10{,}000\times g$). The supernatant was aspirated, pellets were resuspended in 1 ml H/H, recentrifuged, the supernatant discarded, the pellets resuspended in 1 ml H/H, re-centrifuged, the supernatant discarded, and the pellets resuspended in 0.5 ml M/H and stored on ice.

Contact Centrifugation

Four 100 μl aliquots of the cell suspensions were added to wells containing bacteria in flat-bottomed 96-well tissue culture dishes (Costar, Cambridge, MA) sitting on glass plates ($0.6\times8\times12$ cm) on ice. The glass plates facilitated uniform cooling or warming of individual wells. In addition, the glass plates supported the dishes and reduced breakage during contact centrifugation. The wells contained ice cold 1×10^7 cfu of *S. aureus* in 50 μl M/H. The trays (on glass plates) were centrifuged using tissue culture plate carriers at $800\times g$ for 15 minutes at 4°C. Under the stated conditions, contact was accomplished without phagocytosis. At lower centrifugal forces, some bacteria remained in the supernatant. At higher temperatures, phagocytosis and killing was evident within minutes.

Phagocytosis Incubation

The dishes were placed on top of the glass plates on top of a sheet of plate glass in contact with a tray of water in a 37°C incubator. After incubation, usually 30 minutes, the dishes were removed, quick frozen on dry ice, and stored at −20°C for one to three days. There was no loss of bacterial viability after one freeze-thaw as measured by either [^{3}H]thymidine incorporation or colony forming units. Furthermore, one freeze-thaw did not affect the degree of viability even when some killing had been caused by the presence of PMLs, antibiotics, or both. The antibiotics examined were rifampicin, novobiocin, clindamycin, fusidic acid, benzylpenicillin, gentamicin, tetracycline, and vancomycin.

[^{3}H]Thymidine Uptake

After thawing, 50 μl of [^{3}H]thymidine in M/H, 0.025 mCi/ml M/H ([*methyl*-^{3}H]thymidine, Amersham, Arlington Heights, IL), were added to the 150 μl in the wells. The mixture was incubated for 1 hour at 37°C. The radioactive bacteria were collected using a cell harvester (Mash II, Microbiological Associates, Walkersville, MD) equipped with high retention glass fiber filter paper (Type

GF/F, Whatman Laboratory Products, Clifton, NJ). The paper discs were dried, placed in vials containing 10 ml of scintillation counting fluid (Optisol, Isolab Inc., Akron, OH), and the radioactivity determined in a Packard 2660 Scintillation Counter. The results were expressed as the average kcpm (10^3 cpm) of the quadruplicated aliquots ± 1 standard deviation (using N-1). The kcpm were directly proportional to the number of colony forming units confirming the reports of others[24~26].

Sensitivity of *S. aureus* 502A to Antibiotics

Activity in this system requires that the compound possess activity against *S. aureus* 502A. In terms of minimum concentrations required to inhibit bacterial growth (MIC) in M/H, rifampicin was the most potent (MIC 0.06 μg/ml); followed by cephaloridine, benzylpenicillin, novobiocin and fusidic acid (0.25 μg/ml); nargenicin, gentamicin, tetracycline, clindamycin, erythromycin and netilmicin (0.5 μg/ml); dicloxacillin, ampicillin, cefazolin, cephalothin, vancomycin and rosaramicin (1 μg/ml).

The concentrations that inhibited [^{3}H]thymidine uptake by 50% (IC_{50}) after a 1-hour incubation in M/H were the same as the MIC data above with the following exceptions. More activity was apparent with rifampicin (IC_{50} 0.012 μg/ml), clindamycin (0.04 μg/ml), tetracycline (0.2 μg/ml), rosaramicin and vancomycin (0.4 μg/ml). A number of antibiotics were tested that had IC_{50} values of 2 μg/ml: chloramphenicol, kanamycin, dicloxacillin, oxacillin, and nafcillin, none of which were active in the system. IC_{50} values were not determined for a number of compounds that were active in the system. These included lincosaminide analogs, coumarins, paulomycin and tyrocidine.

Results

Effect of Inducing Contact between PMLs and *S. aureus* 502A by Centrifugation

If antibiotic intracellular binding to PMLs was too avid, or if surface-bound antibiotic could not elute, then any extracellular or surface-bound bacteria would be unaffected. This problem would be reduced if contact between the bacteria and the PML-antibiotic complex was assured. Centrifugation of the tissue culture trays (4°C, 15 minutes, $800 \times g$) was employed to accomplish contact between PMLs and bacteria.

The results in Fig. 1 show that centrifugation had no effect on bacteria alone or in the presence of serum. Similarly, in the absence of centrifugation, PMLs with or without serum had little effect on [^{3}H]thymidine uptake. However, after contact centrifugation and 0.5 hour of incubation, PMLs alone reduced [^{3}H]thymidine uptake from 200 to 115 kcpm, and to 16 kcpm in the presence of serum.

The decrease in [^{3}H]thymidine uptake indicated contact was achieved and increased phagocytosis and killing occurred even in the absence of serum. Serum was omitted from future experiments because [^{3}H]thymidine uptake was so low when serum was present with PMLs that there would be little margin for further reduction if antibiotics were present.

In a second experiment, antibiotics were incubated with PMLs, the PMLs were washed and added to trays containing bacteria. One tray was stored (at 4°C) and the other centrifuged. The trays were then incubated at 37°C and the assay performed as usual. The results in Table 1 show that contact

Table 1. Effect of contact centrifugation after incubation of antibiotics with PMLs.

Antibiotic	μg/ml	Control	Contact centrifuged
None	None	62±7* (100%)**	41±5 (100%)**
Rifampicin	1.25	48±13 (77%)	23±5 (56%)
Novobiocin	5	46±3 (74%)	24±2 (59%)
Clindamycin	5	59±6 (95%)	32±2 (78%)
Fusidic acid	5	26±2 (42%)	13±1 (32%)

* kcpm±1 standard deviation (using N-1).

** Percent compared with the corresponding "None" control.

Fig. 1. Effect of inducing contact between PMLs and *S. aureus* 502A by centrifugation.

The black symbols represent tissue culture trays that were centrifuged at 4°C for 15 minutes at 800×*g*; open symbols represent trays allowed to stand on ice for 15 minutes. Both types of trays were incubated at 37°C for the times indicated and then frozen on dry ice. Autologous serum was present at a final concentration of 10%.

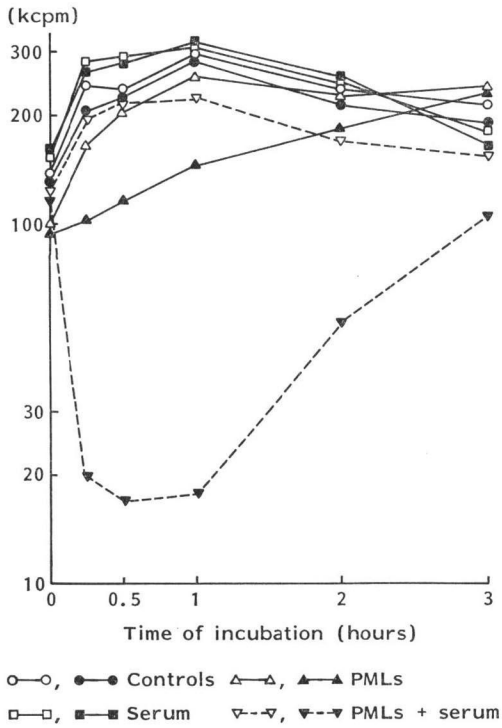
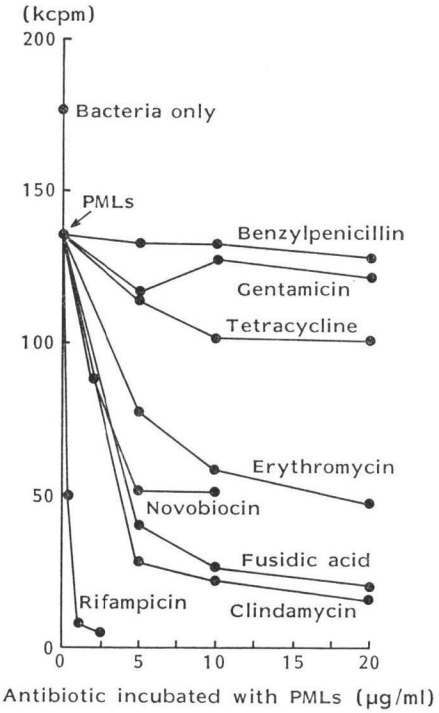


Fig. 2. Antibiotic binding to PMLs.

Antibiotics were incubated at the concentrations shown below with PMLs for 20 minutes at 37°C. The PMLs were washed and the assay performed as described.



centrifugation resulted in greater percent reduction of [^{3}H]thymidine uptake with all four antibiotics tested. The largest difference was seen with rifampicin (77% *versus* 56%). Thus, the sensitivity of the system to antibiotics incubated with PMLs also was increased by contact centrifugation.

Antibiotic Binding to PMLs

Eight antibiotics were incubated with PMLs for 20 minutes at 37°C at the concentrations shown in Fig. 2. Rifampicin, clindamycin, fusidic acid, novobiocin and erythromycin were active. Tetracycline was less active, and no binding could be demonstrated with gentamicin and benzylpenicillin. Dose-response curves were generated with the active antibiotics (with the exception of the highest concentration of novobiocin which, based on other experiments, represented a technical error).

A number of antibiotics were similarly tested at 10 μg/ml for 20 minutes at 37°C but no activity was obtained. These included cephaloridine, nargenicin, netilmicin, dicloxacillin, ampicillin, cefazolin, cephalothin, vancomycin and rosaramicin.

Table 2 shows that many antibiotics were carried by the PMLs. These included paulomycin 273b_1, naphthalenic ansamycins (streptovaricins, rifamycins, tolypomycin Y), coumarins (chlorobiocin and coumermycin), and tyrocidine.

Table 2. Antibiotics that bound to PMLs which decreased [^{3}H]thymidine uptake by *S. aureus* 502A.

Experiment	Antibiotic*	kcpm±1 SD
1	None	130±11
	Paulomycin 273b$_1$	24±9
	Rifampicin	21±2
2	None	140±5
	Streptovaricin A	120±17
	Streptovaricin C	110±6
	Streptovaricin D	86±3
	Rubradirin	42±6
	Rifamycin B	8±1
	Rifamycin S	6±1
	Rifamycin SV	6±1
	Tolypomycin Y	5±1
	Rifampicin	21±12
3	None	128±1
	Chlorobiocin	7±1
	Coumermycin	8±1
	Clindamycin	51±4
4	None	139±8
	Tyrocidine	64±7
	Clindamycin	73±10

* All antibiotics were incubated at 10 μg/ml with PMLs for 20 minutes at 37°C.

Table 3. Lincosaminides that bound to PMLs and decreased [^{3}H]thymidine uptake by *S. aureus* 502A.

Experiment	Compound*	kcpm±1 SD
1	None	116±13
	Clindamycin	53±4
	4′-Pentyl-*N*-demethylclindamycin	15±1
	4′-Pentyl-*N*-β-hydroxyethyl-clindamycin	12±1
	4′-Hexyl-*N*-demethylclindamycin	14±1
2	None	171±13
	Clindamycin	119±9
	N-Methyl-7(*S*)-*S*-methylpirlimycin	100±12
	4′-Butylpirlimycin	55±4
	4′-Hexylpirlimycin	22±1
	4′-Octylpirlimycin	24±2
3	None	139±9
	Clindamycin	74±10
	7-Methylclindamycin	19±1
	N-Ethylclindamycin	21±1

* All antibiotics were incubated at 10 μg/ml with PMLs for 20 minutes at 37°C.

Table 4. The effect of temperature on antibiotic binding to PMLs.

Compound	Concentration (μM)	Incubation temperature		Decrease in kcpm 0°C/37°C
		0°	37°C	
None	—	97±18*	102±23	None
Rifampicin	1.25	104±4	11±1	9.5
Rifampicin	5	42±5	5±1	8.4
Novobiocin	2.5	80±6	38±7	2.1
Novobiocin	10	68±4	14±3	4.9
Fusidic acid	5	80±6	21±1	3.8
Fusidic acid	20	38±3	11±1	3.5
Clindamycin	5	123±10	68±2	1.8
Clindamycin	20	80±8	17±3	4.7

* kcpm±1 SD.

Table 3 shows that several members of the lincosaminide group of antibiotics were active, including both clindamycin and pirlimycin analogs.

The Effect of Temperature on Antibiotic Binding to PMLs

Four antibiotics were incubated with PMLs for 20 minutes at either 0°C or 37°C. As the results in Table 4 show, the binding of all four was greatly reduced at 0°C. Rifampicin binding appeared to be reduced most by the lower temperature.

Antibiotic Binding to Different Blood Cells

Different blood cells were compared for their ability to bind 4 antibiotics (Table 5). PMLs were compared with erythrocytes and mononuclear leukocytes (17% mononuclear phagocytes and 83% lymphocytes). All 4 antibiotics bound to about the same degree to both mononuclear and poly-

Table 5. Antibiotic binding to PMLs, mononuclear leukocytes, and erythrocytes.

Antibiotics*	kcpm±1 SD		
	PML	MNL	RBC
None	160±13	169±14	186±18
Rifampicin	7±2	4±1	144±10
Novobiocin	48±4	30±3	185±10
Fusidic acid	75±11	63±5	164±17
Clindamycin	118±5	131±9	186±18

* Antibiotics, 10 μg/ml, were incubated with 2.5×10^6 of each of the blood cell types shown above at 37°C for 20 minutes.

Table 6. Reversal of clindamycin binding.

Compounds*	kcpm±1 SD
Control	174±11
Clindamycin only	52±10
Clindamycin+proline amide	143±9
Clindamycin+proline benzyl ester	160±6
Clindamycin+puromycin	154±5
Clindamycin+adenosine	111±23

* The compounds were present at a final concentration of 1 mM; clindamycin at 0.02 mM. The solutions were incubated with 2.5×10^6 PML per ml at 37°C for 15 minutes. The data are based on the average of three separate PML preparations.

morphonuclear leukocytes. A small but significant amount of rifampicin appeared to be bound to erythrocytes; however, this could be due to contamination of the erythrocyte preparation by small numbers of leukocytes.

Applicability of the System in Studying Inhibitors of Antibiotic Binding

Clindamycin was used to study the applicability of this system in studying compounds that inhibit antibiotic uptake. Certain nucleosides, puromycin and adenosine, have been reported to inhibit the uptake of radioactive clindamycin by alveolar macrophages[4], while proline derivatives such as proline amide and proline benzyl ester inhibited radioactive clindamycin uptake by PMLs[27].

The results of adding these compounds (1 mM) to clindamycin (0.02 mM) on binding are shown in Table 6. Proline amide, proline benzyl ester, and puromycin reversed clindamycin binding; adenosine was somewhat less effective. It should be noted that these reversing effects were not seen if the compounds were first incubated with PMLs, the PMLs washed twice, and then incubated with clindamycin. This shows that the compounds did not inhibit clindamycin binding through toxic mechanisms as did some compounds (*e.g.*, proline-β-naphthalamide).

Discussion

The system described here represents another method by which interactions between antibiotics and leukocytes can be measured. The primary advantages of the system are the opposite of the limitations of three classical systems. Thus, to display activity in this system, the antibiotic does not need to be radiolabeled, it does not have to kill intracellular microbes, nor does it need to affect any phagocyte function. Other advantages are that large numbers of a variety of antibiotics can be screened, it is sensitive to antibiotic concentrations likely to be found in patient's sera (10 μg/ml or less), and it can be used to study mechanisms of binding.

The primary limitations of the system are that specific antibiotic activity against the test organism is required, and the antibiotic must bind tight enough to remain attached to the leukocyte through two washings. These limitations notwithstanding, the fact that the results obtained with this system mirror those obtained in the other three systems provides evidence for its validity in studying antibiotic-phagocyte interactions. First, the current results confirm previous positive binding results using radioactive rifampicin[1,2], clindamycin[2~5], erythromycin[5] as well as negative results obtained with gentamicin, benzylpenicillin, and cefazolin[6]. In addition, the binding of radiolabeled rifampicin and clindamycin had been shown to be temperature dependent[3,5]. These results were confirmed and it was shown that the binding of novobiocin and fusidic acid also was temperature dependent.

Second, the data obtained here corroborate that obtained previously with rifampicin which can penetrate and kill staphylococci inside phagocytes[1,6,7,11]. Recently, other naphthalenic ansamycins were found to possess similar properties[14]. Thus, it was likely that streptovaricins, other rifamycins, and tolypomycin Y might be active in the binding system and this hypothesis was confirmed.

Third, antibiotics which have been reported to affect some leukocyte function such as chemotaxis were also active in the binding system. These included tetracycline[15~17], rifampicin[18~20], fusidic acid[18,19], tyrocidine[21], and clindamycin[22].

The binding of radioactive clindamycin has been shown to be inhibited by puromycin and adenosine[4] and by certain proline derivatives[27]. These results were repeated suggesting that the current system can be used in standard ligand-receptor pharmacologic studies.

Antibiotics that have not been reported previously (to our knowledge) to interact with PMLs included paulomycin; rubradirin; the coumarins novobiocin, coumermycin, and chlorobiocin; the naphthalenic ansamycins streptovaricins A, C, and D, rifamycins B, S, and SV, tolypomycin Y; and a variety of lincosaminides including clindamycins and pirlimycins. Thus, the system was used to examine many types of antibiotics.

The clinical relevance of knowing that an antibiotic binds to leukocytes is not clear. Binding might be due to simple dissolution into plasma membranes so than an antibiotic might bind in a non-specific fashion to a wide variety of cells and tissues. Moreover, either non-specific or specific binding could be detrimental by causing a reduction in bioavailable antibiotic. Nevertheless, specific antibiotic binding to phagocytes could be important because binding is the first step in antibiotic penetration which has obvious implications in combatting pathogens residing within leukocytes as well as in treating infections characterized by abscesses and granulomas. The system described here might be useful as a preliminary test for finding antibiotics that possess these additional useful properties.

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