ANTIMICROBIAL AGENTS

Gentamicin Inactivation in Purulent Exudates: Role of Cell Lysis

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Factors contributing to the binding and reversible inactivation of gentamicin by purulent exudates were studied in a simplified in vitro model consisting of purified human polymorphonuclear leukocytes (PMNLs). Whereas intact PMNLs (10^6–10^8/ml) bound almost no [^{14}C]gentamicin, freeze-thawed PMNLs showed extensive [^{14}C]gentamicin binding, expressed as antibiotic cosedimenting with particulate material from the lysed PMNLs. Antibiotic binding could be related to the concentration of lysed PMNLs and to the amount of [^{14}C]gentamicin added. Binding of [^{14}C]gentamicin by lysed PMNLs was highly sensitive to DNase I but was unaffected by RNase, Triton X-100, or protease. Purified chromatin or DNA from either purulent exudates or lysed PMNLs reproduced the [^{14}C]gentamicin-binding pattern obtained with crude PMNL lysate. These results show that gentamicin inactivation in purulent exudates can be correlated with binding of the antibiotic to lysed PMNLs; PMNL chromatin DNA is identified as one of the major binding factors.

Successful control of bacterial infections requires besides other factors the adequate diffusion of antibiotics to the sites where the pathogenic microorganisms reside. Antimicrobial agents are known to bind to, and to be partially and reversibly inhibited by, a variety of tissue constituents, including interstitial fluid proteins, cell membranes, soluble intracellular proteins, and particulate intracellular components [1–3]. Thus, such sedimentable components have been shown to bind avidly gentamicin and polymyxin in purulent exudates, leading to a considerable reduction of the concentration of the free, biologically active drugs [4].

The mechanisms leading to the reversible inactivation of aminoglycosides by purulent exudates are largely unknown. In order to investigate this inhibition under controlled conditions, we first established an in vitro model of a purulent exudate, consisting exclusively of intact, purified, human polymorphonuclear leukocytes (PMNLs) suspended in a physiological buffer [5]. Under

such conditions, no binding and/or inactivation of gentamicin by either resting or actively phagocytizing PMNLs could be demonstrated, and the decreased antibacterial effect of the aminoglycoside could be ascribed to its poor penetration into the intact phagocytes, which were probably playing the role of transient bacterial sanctuaries [5].

Purulent exudates, however, consist of intact as well as lysed inflammatory cells in various proportions; it was observed in the experiments described above that disruption of the PMNLs led to a considerable binding of gentamicin to the cellular components. In vitro lysis of PMNLs therefore turned out to be a convenient, well-controlled system for the analysis of various parameters influencing the binding and inactivation of aminoglycosides and for the exploration of their binding sites at the subcellular level. In the present study, we define some of the factors influencing this binding reaction and demonstrate that it can largely be accounted for by aminoglycoside–leukocyte chromatin interaction. Finally, we show that the gentamicin-binding activity of lysed, purified PMNLs occurs to the same extent as aminoglycoside binding with fresh, native purulent exudates from various origins.

Materials and Methods

Antibiotics. [*Methyl-*^{14}C]gentamicin   sulfate

(specific activity, 1,842 decays per min/μg) was a gift from Schering Corp., Bloomfield, N.J. An aqueous stock solution of 1,000 μg of the free base/ml was prepared and stored at 4 C without any significant loss of bioactivity. Injectable sisomicin (Schering); amikacin and ampicillin (Bristol Meyers S.A., Zug, Switzerland); methicillin and carbenicillin (Beecham Pharmaceuticals, Betchworth, England); tobramycin, vancomycin hydrochloride, and sodium cefazolin (Eli Lilly, Indianapolis, Ind.); and penicillin G (Mycofarm, Delft, the Netherlands) were freshly prepared as solutions containing 1 mg of antibiotic/ml of saline for each assay. Pure clindamycin phosphate and rifampicin, donated by Upjohn (Zürich, Switzerland) and Lepetit (Milan, Italy), respectively, were also solubilized at a concentration of 1 mg/ml, but in distilled water (clindamycin) or in 0.01 N HCl (rifampicin).

Preparation of leukocytes. Purified PMNLs, or lymphomonocytes, were purified by the technique of Böyum [6]. Aliquots of the leukocyte preparation, containing the required number of PMNLs or lymphomonocytes (see below), were counted in a hemacytometer and distributed in a set of preweighed, conical sterile centrifuge tubes. Leukocytes were sedimented for 10 min at 3,000 g and suspended in 0.9 ml of a Dulbecco's phosphate-buffered saline (PBS) solution (Gibco Bio-Cult, Glasgow, Scotland), adjusted to pH 6.0. Unless otherwise indicated, lysed leukocyte suspensions were obtained by freezing for at least 18 hr at −20 C followed by thawing at 37 C. This procedure led to a complete loss of cell viability [7] as demonstrated by a >99% permeability to trypan blue.

Purification of chromatin. A simple procedure for isolation of interphase chromatin structures from cultured cells [8] was found to be convenient for the purification of chromatin from PMNLs. In brief, 10^8 PMNLs were allowed to swell at 4 C in a hypotonic solution containing 0.1 M sucrose in 0.2 mM phosphate buffer (pH 7.5) for 2 min. An equal volume of ice-cold detergent solution containing 0.5% Nonidet® P 40 (NP 40; Shell Chemical Corp., New York, N.Y.) in 0.2 mM EDTA (pH 7.5) was added by drops, at a rate of 1 ml/min, leading to PMNL lysis, as evidenced by the demonstration under a microscope of numerous chromatin bodies. After dispersion of the chromatin bodies (which showed a strong tendency to aggregate), the suspension was separated from the cell lysate by layering over a 0.1 M sucrose-0.2 mM phosphate buffer solution, pH 8.5, and by centrifugation at 3,500 g for 20 min.

Binding of [^{14}C]gentamicin to PMNL lysate: assay of antibiotic and DNA determinations. Binding of [^{14}C]gentamicin was expressed as the amount of antibiotic cosedimenting (3,000 g for 10 min) with particulate material from lysed PMNLs. The incubation conditions were as follows: 0.1 ml of the aqueous stock solution of [^{14}C]gentamicin, containing 100 μg of free base (240,000 decays per min), was added to 0.9 ml of a suspension in PBS of either lysed PMNLs or purified chromatin structures. Each sample was incubated for 1 hr at 37 C before centrifugation at 3,000 g for 10 min. The first supernatant containing the unbound [^{14}C]gentamicin was carefully separated from the wet pellet; its contribution to the total radioactivity of the wet pellet was quantitated by the procedure described below. Radioactivity of the supernatant and of the pellet was estimated in a liquid scintillation counter (LS 3135-T; Beckman Instruments, Irvine, Calif.); 0.1-ml aliquots of the supernatant were emulsified in 5 ml of the scintillator fluid Dimilume® 30, which contains a chemiluminescence inhibitor (Packard Instrument Co., Downers Grove, Ill.), and counted. The [^{14}C]gentamicin present in the wet pellet was quantitatively extracted in a 5% solution of trichloroacetic acid (TCA), and 0.1-ml aliquots were emulsified in 5 ml of Dimilume 30 and counted. The counting efficiency was optimal (90%), and the cpm data were directly converted into ng of [^{14}C]gentamicin using the appropriate conversion factor. The extraction step in TCA was carried out at 90 C for 10 min because it extracted simultaneously the nucleic acids [9] of the samples, which were assayed for DNA by the method of Dische [10]. After heating, the samples were cooled at 4 C for 15 min before centrifugation at 3,000 g for 10 min.

For small chromatin fragments, the following modifications of the antibiotic-binding assay were introduced: at the end of the 60-min incubation at 37 C with [^{14}C]gentamicin in PBS, the chromatin fragments or the purified DNA was sedimented at 290,000 g for 120 min at 4 C. The pellet was first suspended in TNE (20 mM Tris-HCl, 40 mM NaCl, 1 mM EDTA; pH 7.5), to which one volume of 10% TCA was added. This time, extraction of [^{14}C]gentamicin was carried out in the cold (4 C) because the suspension was too dilute to allow

detection of DNA by the colorimetric method [10]. After precipitation in cold 5% TCA, the sedimented (3,000 g for 10 min) nucleic acids were selectively extracted in 0.5 ml of 5% TCA at 90 C for 10 min and assayed colorimetrically [10] for DNA.

The reproducible, full recovery of [^{14}C]gentamicin in the supernatants and in the pellet extracts also required an accurate volumetric estimation of the fluids containing the radioactive antibiotic. This estimation was done by gravimetry, since the density either of supernatant fluid or of the TCA pellet extract was close to that of water (<1.02).

Volumetric estimation of the wet pellet was also done by gravimetry. The contribution of contaminating supernatant fluid to the wet pellet was estimated by the formula $W_{SN} = W_{WP} - W_D$, where W_{SN} is the weight of supernatant fluid, W_{WP} is the weight of the wet pellet, and W_D is the weight of the heat-dried pellet. The value of W_D turned out to be ~1 mg for 10^7 leukocytes. When intact leukocytes were tested in the binding assay, the contribution of their intracellular fluid to the weight of the wet pellet was also taken into consideration in estimating the weight of contaminating supernatant fluid according to the modified formula $W_{SN} = W_{WP} - W_D - W_{IF}$, where W_{IF} is the weight of intracellular fluid. This value has been shown to be 2 mg/10^7 leukocytes [11].

Binding assay with nonradioactive antibiotics. Suspensions (0.9 ml) of 5 × 10^7 lysed PMNLs in PBS were supplemented with 0.1 ml of a 10-fold concentrated solution of the nonradioactive antibiotic to be tested. The conditions of incubation and of sedimentation were identical to those used in the [^{14}C]gentamicin-binding assay. The supernatant and the pellet, suspended in PBS, were assayed microbiologically using a modified agar-well method [12] and *Bacillus subtilis* ATCC 6633 as the test organism for all of the antibiotics tested, except for carbenicillin (*Pseudomonas aeruginosa* NCTC 10490) and cloxacillin, clindamycin, and rifampicin (*Sarcina lutea* NCTC 8340). Standard curves were obtained by dilution of the antibiotics in PBS.

Enzyme or detergent pretreatment of lysed PMNLs and of purified chromatin. Lysed PMNLs (10^7) or purified chromatin at an equivalent DNA content was sedimented at 3,000 g for 10 min, suspended, and incubated for 60 min at 37 C with one of the following enzymes or detergent: (*1*) pancreatic DNase I (Serva Feinbiochemica, Heidelberg, West Germany), 50 μg/ml in 10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl$_2$ (pH 7.4); (*2*) the same buffer without DNase; (*3*) pancreatic RNase A (Serva Feinbiochemica), 20 μg/ml in 10 mM Tris-HCl, 10 mM NaCl (pH 7.4); (*4*) protein-
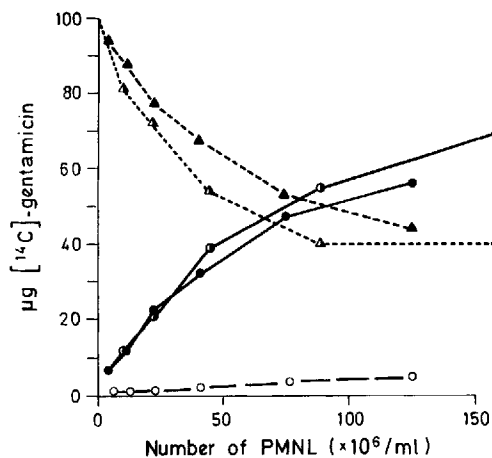

Figure 1. Binding of 100 μg of [^{14}C]gentamicin in a 1-ml assay by purified intact (O) or freeze-thawed polymorphonuclear leukocytes (PMNL) as a function of cell concentration: (●) = sediment; (▲) = supernatant. Similar binding values were obtained with a native, purulent empyema, adjusted for an equal number of trypan blue–positive PMNL: (⊙) = sediment; (▲) = supernatant.

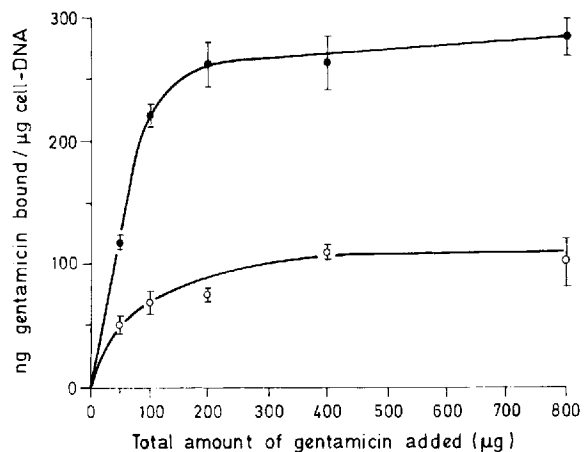**Figure 2.** Binding of [^{14}C]gentamicin in a 1-ml assay by sediments of 10^7 purified freeze-thawed polymorphonuclear leukocytes (PMNLs) (●) or by purified chromatin from 10^7 PMNLs (O), suspended in phosphate-buffered saline as a function of the total amount of antibiotic added. All binding values are expressed as ng of gentamicin bound/μg of leukocyte DNA; each value represents the mean of triplicate assays ± SEM.

ase K (Merck, Darmstadt, West Germany), 100 μg/ml in 5 mM Tris-HCl, 5 mM NaCl, 5 mM EDTA (pH 8.0); (5) 0.2% Triton X-100 (Merck) in 5 mM Tris-HCl, 5 mM NaCl, 5 mM EDTA (pH 8.0). After incubation, the residual material was sedimented for 10 min at 3,000 g and resuspended in 0.9 ml of PBS. An aliquot (0.1 ml) of the 1 mg of [^{14}C]gentamicin/ml stock solution was added to each tube, and the binding assay was performed as described above.

Statistical analysis. Each individual assay condition was tested in triplicate tubes, with differences in results analyzed by Student's *t*-test.

Results

Gentamicin binding of lysed peripheral PMNLs and of PMNLs obtained from purulent exudates. The gentamicin-binding activities of PMNLs incubated under different conditions in PBS are summarized in figure 1. Whereas intact, freshly collected PMNLs bound almost no [^{14}C]gentamicin, lysed PMNLs showed extensive binding, which was dependent on the number of lysed cells added to the system. While 10^8 lysed PMNLs bound 50 μg of gentamicin in a 1-ml assay, almost identical results were obtained with a native preparation of a purulent exudate containing the same number of trypan blue–positive

PMNLs. Binding of the aminoglycoside by lysed, purified PMNLs or empyema at various concentrations was corroborated by the concomitant decrease in [^{14}C]gentamicin concentration in the supernatant fluid. The same results were obtained with a preparation of purified, lysed lymphocytes and monocytes (data not shown). Control experiments, performed either with a suspension or with a washed sediment of lysed PMNLs, showed identical gentamicin binding; therefore, further binding assays were performed with leukocytic sediments washed in PBS.

Gentamicin-binding conditions in the presence of lysed PMNLs. In the presence of a constant amount of a leukocyte sediment equivalent to 10^7 lysed PMNLs, [^{14}C]gentamicin binding in the presence of increasing concentrations of the antibiotic was a saturable process (figure 2). From 0.75 μg to 100 μg of [^{14}C]gentamicin/ml, the amount of antibiotic bound to the leukocytic sediment was proportional to the total amount of gentamicin added (eight concentrations tested, data not shown), whereas with >200 μg/ml the binding process was saturable. To correct for the occasional variability in the PMNL counts of the samples and to correlate the binding results with those obtained in presence of chromatin (see below), all of the binding values were expressed as a function of the DNA content of the sedimented, lysed

Table 1. Binding of various antibiotics by lysed human polymorphonuclear leukocytes (PMNLs).

	Amount of antibiotic (μg)			
Antibiotic	Total added	Bound to sediment*	Bound to supernatant	Total recovered
Gentamicin	10	3.7 ± 0.8	6.3 ± 0.1	10.0 ± 0.8
Tobramycin	10	4.6 ± 1.5	5.0 ± 0.04	9.6 ± 1.5
Sisomicin	10	3.7 ± 0.7	6.3 ± 0.1	10.0 ± 0.7
Neomycin	10	4.6 ± 1.5	3.0 ± 1.0	7.6 ± 0.8
Amikacin	20	1.8 ± 0.2	15.1 ± 1.0	16.9 ± 1.2
Penicillin G	100	1.6	99.0	100.6
Ampicillin	100	1.1	93.0	94.1
Methicillin	100	0.1	90.0	90.1
Cloxacillin	100	2.6	95.0	97.6
Carbenicillin	100	<6.0†	101.0	<107.0
Cefazolin	100	0.3	91.0	91.3
Vancomycin	100	7.9	89.6	97.5
Vancomycin	10	1.8	7.3	9.1
Clindamycin	10	<1.0†	11.0	<12.0
Rifampicin	10	1.3	10.8	12.1

NOTE. See Materials and Methods for procedures used in the binding assay and the microbiological assay for antibiotic. Each binding assay was performed in a total volume of 1 ml. Where indicated, data are means of triplicate assays ± SEM.

* Each sediment corresponded to 5 × 10^7 lysed PMNLs.

† The sensitivity limit of the microbiological assay.

Table 2. Binding of [¹⁴C]gentamicin (100 μg/ml) by lysed polymorphonuclear leukocytes (PMNLs) (10⁷/ml) pretreated with various enzymes or nonionic detergent.

| Pretreatment | Lysed PMNL sediment | | [¹⁴C]gentamicin bound (ng)/DNA content (μg) |
	[¹⁴C]gentamicin bound (μg)	DNA content (μg)	
None	8.79 ± 0.78	73.7 ± 2.0	119 ± 11
RNase A	8.80 ± 0.32	70.3 ± 2.7	125 ± 4
Triton X-100	8.86 ± 0.46	65.5 ± 3.1	135 ± 4
Proteinase K	11.87 ± 0.50	77.1 ± 14.4	154 ± 43
DNase I	1.60 ± 0.26*	25.1 ± 3.1*	64 ± 16

NOTE. See Materials and Methods for preincubation and binding assay procedures. Data are means of triplicate assays ± SEM.
* *P* < 0.001 compared with untreated sediment.

PMNLs; thus, 10⁷ lysed PMNLs had a mean DNA content of 70 μg and bound 17 μg of gentamicin under saturating conditions.

Binding/inhibition of other antibiotics by lysed PMNLs. Besides gentamicin, a number of other antibiotics was tested by microbiological assay for their binding by lysed PMNLs (table 1). Binding and inhibition by 5 × 10⁷ lysed PMNLs could be demonstrated mainly with antibiotics of the aminoglycoside group. None of the penicillins or cephalosporins tested showed any binding with the leukocytic sediments, and little inactivation was observed with vancomycin and rifampicin. Finally, fully biologically active aminoglycosides could be recovered from the sediment (table 1), a result demonstrating the reversibility of the inactivation process.

Identification of PMNL substrates responsible for aminoglycoside binding. In an attempt to define the PMNL substrates with gentamicin-binding activity, the leukocyte sediments were predigested with various enzymes or nonionic detergents and tested for residual gentamicin-binding activity (table 2). Neither RNase nor Triton X-100 nor proteinase K removed any binding activity

from the leukocytic sediments; in comparison, pancreatic DNase I decreased the activity by >80% (*P* < 0.001). DNA assay of the residual material after the various predigestions indicated that 66% of the DNA originally present in the leukocytic sediment had been digested by DNase I (*P* < 0.001), in contrast to the lack of effect of the other agents. Incomplete digestion of chromatin DNA by DNase I is thought to have resulted from its protection by chromatin proteins [13] and probably explains the residual 20% binding activity of the leukocytic sediment after digestion.

To assess further the correlation between the role of DNA-containing material and its gentamicin-binding activity, purified PMNL chromatin was prepared and incubated in the presence of labeled antibiotic. For a constant amount of purified chromatin (figure 2, bottom curve) extracted from 10⁷ PMNLs, [¹⁴C]gentamicin binding as a function of aminoglycoside concentration exhibited the same saturation process previously demonstrated with the same number of lysed PMNLs (figure 2, top curve). Although the two systems were saturated at the same value of total gentamicin added, the purified chromatin samples

Table 3. Binding of [¹⁴C]gentamicin (100 μg/ml) by the purified chromatin fraction obtained from polymorphonuclear leukocytes (10⁷/ml) pretreated with various enzymes.

| Pretreatment | Chromatin | | [¹⁴C]gentamicin bound (ng)/DNA content (μg) |
	[¹⁴C]gentamicin bound (μg)	DNA content (μg)	
None	5.52 ± 0.11	51.3 ± 0.3	108 ± 3
RNase A	5.33 ± 0.10	57.6 ± 0.3	92 ± 1
Proteinase K	7.62 ± 0.40*	65.6 ± 1.2	116 ± 5
DNase I	3.78 ± 0.10*	36.3 ± 2.4†	104 ± 7
Sonication plus DNase I	0.12 ± 0.04*	11.3 ± 2.4*	11 ± 1*

NOTE. See Materials and Methods for the preincubation and binding assay procedures. Data are means of triplicate assays ± SEM.
* *P* < 0.001 compared with untreated, purified chromatin.
† *P* < 0.005 compared with untreated, purified chromatin.

bound only 40% of the gentamicin bound by lysed PMNLs under saturating conditions. Such lowered efficiency of gentamicin-binding activity exhibited by the purified chromatin fractions probably resulted from their highly aggregated state during incubation in PBS for the binding assay.

Purified chromatin was also digested by different enzymes as described previously for lysates of PMNLs (table 3). Neither RNase A nor proteinase K decreased DNA content or binding activity of the purified chromatin. DNase I was less active on purified chromatin in its aggregated state than on leukocyte sediments; thus, 68% of the chromatin DNA resisted digestion by DNase I, and 71% of the [¹⁴C]gentamicin-binding activity was still present at the end of the preincubation phase. Brief successive treatments of the purified chromatin with DNase I for 5 min improved the DNA digestion, with >80% of the DNA being removed, accompanied by a similar loss in gentamicin-binding activity (duplicate assays, data not shown). Finally, the solubilization of purified chromatin was improved by brief sonication and centrifugation at high speed (290,000 g for 2 hr); the sonicated chromatin pretreated with DNase I lost 97% of its gentamicin-binding activity, while DNA assays of the high-speed pellets indicated that 90% of the chromatin DNA had been hydrolyzed (table 3).

These results taken together suggested that the DNA fraction is essential for the gentamicin-binding activity of purified chromatin. In contrast, the presence of chromatin histone and nonhistone proteins was of minor importance since their selective extraction by either 0.2 N H_2SO_4 or 0.4 M NaCl did not remove any gentamicin-binding activity (data not shown).

Correlation between gentamicin-binding activity of lysed PMNLs and of native, purulent exudates. To correlate gentamicin binding of lysed PMNL suspensions with that of naturally occurring purulent exudates, three of such exudates obtained from three patients were incubated at a concentration of 2–10 × 10⁷ cells/ml with 10 μg or 100 μg of gentamicin under various conditions (table 4). Binding to sedimentable material was in good agreement with that demonstrated in figure 1 and could be further increased by incubation in hypotonic medium or by freeze-thawing. Finally, the role of DNA as a main binding substrate of gentamicin in a fresh, native, purulent exudate was confirmed by the demonstration that purified DNA from purulent exudate from patient no. 1, when preincubated with 100 μg of [¹⁴C]gentamicin for 60 min and ultracentrifuged at 290,000 g for 120 min, exhibited a binding of 18 μg of the antibiotic to 62 μg of purified DNA. The ratio of 290 ng of [¹⁴C]gentamicin bound/μg of purified DNA

Table 4. Cellular binding of [¹⁴C]gentamicin by purulent exudates: role of cell lysis and of incubation conditions.

Patient no./origin (microorganism)	Leukocyte count	Leukocytes excluding trypan blue (%)	[¹⁴C]gentamicin (μg)		
			Total added	Recovered in native PMNL sediment	Recovered in freeze-thawed PMNL sediment
1, perianal abscess (*Staphylococcus aureus*)	10 × 10⁷	90	100*	13.9	33.0
2, pleural empyema (anaerobes)	10 × 10⁷	60	100*	9.6	34.0
			10*	2.4	4.8
3, abdominal abscess (*Escherichia coli*)	2 × 10⁷	ND	100†	8.2	15.0
			100‡	30.8	35.8
Purified circulating human PMNLs	1 × 10⁷	100	100†	1.0	9.8
			100‡	15.9	16.1

NOTE. Each binding assay was performed in a total volume of 1 ml. PMNL = polymorphonuclear leukocyte; ND = not done.

* Uncentrifuged exudate.
† Washed and suspended in phosphate-buffered saline at its original concentration.
‡ Washed and suspended in distilled water at its original concentration.

measured was in good agreement with that obtained with lysed PMNLs under saturating conditions (figure 2).

Discussion

During the last decade, increased interest has developed in the penetration of antibiotics into normal and infected tissues, in order to delineate possible factors leading to therapeutic failure in localized infections [1]. Such studies have been hampered by various methodological problems, such as the complex constitution of tissue homogenates, the individual characteristics of each antibiotic, and the influence of various physicochemical parameters [2].

As a special case of interaction between antibiotic and tissue constituents, Bryant and Hammond [4] have recently demonstrated in a simple assay system that aminoglycosidic antibiotics bind avidly, but reversibly, to a homogenized purulent exudate. Such an interaction, resulting in partial fluid-phase inactivation of the antibacterial agents, offered a plausible explanation for the difficulty in sterilizing pseudomonas abscesses [4]. These studies, although contributing to our understanding of the fate of aminoglycosidic antibiotics in purulent exudates, did not delineate either the binding conditions or the binding substrate(s) of such exudates, although intact or lysed PMNLs or subcellular fractions thereof were candidates to be considered [2–4].

In a previous study [5], we were able to demonstrate that gentamicin bound poorly to highly purified, intact human PMNLs. In the present study, we provide evidence that an "artificial purulent exudate," obtained by lysis of human PMNLs under well-controlled conditions, binds gentamicin and other aminoglycosidic antibiotics by virtue of its high content in human PMNL-derived chromatin. The adequacy of our in vitro model as a paradigm of a purulent exudate for these experiments is suggested by the identical gentamicin-binding curves obtained by equal numbers of either purified, lysed PMNLs or leukocytes obtained from a native exudate incubated under identical conditions (figure 1, table 1); gentamicin-binding activity of purulent exudates, centrifuged and resuspended in PBS and assayed without freeze-thawing, was superimposable on that obtained with purified PMNL sediments lysed to obtain an equivalent number of trypan blue–positive cells.

Before reviewing the chromatin-binding experiments, several aspects of our in vitro cellular model should be discussed. (*1*) As shown in table 1 and figure 1, binding of aminoglycosides to lysed PMNL sediments of pus correlated with an equivalent decrease in antimicrobial activity of the supernatants, an observation indicating that there was no additional soluble inhibitory factor(s) involved in our system. Thus, binding to soluble acid mucopolysaccharides [14] was negligible in our system. (*2*) Binding of [^{14}C]gentamicin to lysed PMNLs or pus resulted in an equivalent loss of its antimicrobial activity, and antibacterial activity could be restored by extraction of the counts from the pellet. The results of these experiments confirmed that the binding process was reversible, as was suggested by the results of Bryant and Hammond [4] under less physiologic conditions. (*3*) The sensitivity of the radioactive assay as well as the intensity of the gentamicin binding to lysed PMNLs allowed us to perform most studies at a low cell concentration. This last point had several advantages, including a more accurate gravimetric estimation of the packed leukocyte volume (their dry weight not exceeding 1 mg), a better control of the ionic strength of the incubation solution, and an easily attainable saturation concentration of gentamicin. Finally, Ca^{++} and Mg^{++} liberated from lysed PMNLs could not influence our microbiological assays of the various aminoglycosidic antibiotics tested, since their contribution of the incubation was <1 microequivalent/10^8 lysed cells.

As shown in figures 1 and 2, binding of gentamicin by lysed PMNLs or native purulent exudate could be reproduced by incubation of gentamicin with chromatin isolated from an equal number of purified PMNLs. Although the curves showed similar saturation kinetics, reaching their plateaus at a gentamicin concentration of 200 μg/ml, the binding activity of the purified chromatin was 40% of the values obtained in the corresponding PMNL sediment for an equivalent DNA concentration. Since purified chromatin was highly aggregated in PBS, as was shown by other investigators [8], it might have reduced the number of gentamicin-binding sites under saturating conditions; alternatively, purification of chromatin might have slightly reduced the binding affinity of the preparation. Additional circumstantial evi-

dence of the important role played by the DNA-containing chromatin material in the binding of [14C]gentamicin was obtained by showing that only DNase treatment, but not incubation with pronase, RNase, or Triton X-100, abolished the cosedimentation of the antibiotic either with lysed PMNLs or with purified chromatin. Direct demonstration of gentamicin binding to DNA-containing material was obtained by incubation of the DNA, purified from the purulent exudate used in figure 1, with the antibiotic, followed by ultracentrifugation. These results are in accord with previous observations; aminoglycosidic antibiotics such as streptomycin bind avidly to DNA and can be used as their precipitating agents [15, 16].

The clinical relevance of the present and previous observations is as yet not fully defined [4, 16]. Binding of aminoglycosides was substantially higher in a study by Bryant and Hammond than in ours, probably as a result of laboratory manipulations such as homogenization and resuspension in hypotonic medium [4]; these factors influenced dramatically the degree of binding of native, purulent exudates, as shown in table 4. Nevertheless, a 10%–30% inactivation of aminoglycosides by a native, purulent exudate could have important consequences in association with other factors present in inflammatory exudates such as low pH and low Po_2, both of which are known to affect adversely the antibacterial effect of aminoglycosides [17, 18]. Experiments performed on an animal model may help to clarify these aspects.

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