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Gentamicin Antibacterial Activity in the Presence of Human Polymorphonuclear Leukocytes

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Complete protection of *Staphylococcus aureus* Wood 46 from gentamicin bactericidal activity was documented for microorganisms located within polymorphonuclear leukocytes. The highest, still ineffective gentamicin concentration tested in the phagocytic assay was 80 times higher than the minimal concentration required to kill uningested organisms. Extracellular gentamicin activity was unaffected by the phagocytic process as demonstrated by microbiological and enzymatic assays, and liberation of intracellular *S. aureus* by lysis of neutrophils showed the bacteria to be fully susceptible to the antibiotic. These results were corroborated by studies performed with [^{14}C]gentamicin; binding of the labeled antibiotic by resting neutrophils, or by neutrophils ingesting live, killed *S. aureus* or endotoxin-coated paraffin particles, showed no statistical differences and never exceeded 20% of the extracellular concentration. These results show that intraleukocytic *S. aureus* are protected from the bactericidal action of gentamicin and suggest that this protection can be explained by poor intracellular penetration of the antibiotic.

Phagocytic uptake of opsonized bacteria, followed by their intracellular killing, is an important defence mechanism against several types of invasive bacterial infections (25). These two groups of events, ingestion and intracellular killing, can be tested independently and have been shown to proceed at different time rates (25). Thus, many virulent *Staphylococcus aureus* are promptly phagocytosed by polymorphonuclear leukocytes (PMN), but remain viable while intracellular for a prolonged period of time (16). The suggestion that such intracellular bacteria might be protected from the bactericidal effect of extracellular II products was already made in 1916 (17), and was thereafter confirmed experimentally in phagocytic systems for several antibiotics (1, 10, 13, 14, 20, 22).

Although aminoglycosidic antibiotics such as streptomycin or gentamicin are potent bactericidal agents against *Staphylococcus aureus* in a cell-free in vitro system, their effectiveness in clinical infections is by far not convincing (9, 12). Emergence of variant forms after exposure to gentamicin offers only a partial explanation of this discrepancy, since their demonstration is rare in clinical isolates and inconstant during laboratory manipulation (15). Direct evidence of the protection of *S. aureus* from gentamicin in the presence of phagocytic cells was therefore looked for in the present study by two different techniques. First, viability of ingested organisms in the presence of bactericidal concentrations of gentamicin was shown to be unaffected by the antibiotic. Second, binding or penetration of radioactive gentamicin to purified PMN or both was found to be low and unaffected by active ingestion of live bacteria or inert particles. These results demonstrate the complete protection of ingested *S. aureus* from the bactericidal effect of gentamicin and allow us to postulate insufficient intraleukocytic penetration or availability of the antibiotic as one of the responsible mechanisms.

METHODS

Bacterial strains. *Staphylococcus aureus* strain Wood 46 was used in the majority of the phagocytic experiments, its opsonic requirements being limited to alternative pathway-mediated complement activity (11). The strain used was susceptible to gentamicin at a concentration of 0.1 μg/ml in Mueller-Hinton broth. For the experiments measuring the binding of radioactive gentamicin, another gentamicin-resistant strain had to be used: this clinical isolate, obtained at the Hôpital Cantonal Geneva and called MR-GR 1, was found to be also resistant to methicillin, erythromycin, tetracycline, and kanamycin. A uniform subpopulation of strain MR-GR 1, composed exclusively of cells able to grow in presence of 100 μg of gentamicin per ml, was used for the binding experiments and was obtained by an overnight exposure to 100 μg of gentamicin per ml in Mueller-Hinton broth at 37°C. This strain did not inactivate gentamicin in vitro.

To rule out that preincubation of *S. aureus* in the presence of gentamicin might influence the extent of binding of [^{14}C]gentamicin, identical binding experiments were run with MR-GR 1 without preincubation

and with strain Wood 46. No statistical difference could be demonstrated among the three experimental conditions, the Wood strain showing slightly less binding to and being rapidly killed by the aminoglycoside.

Chemicals and media. [*methyl*-^{14}C]gentamicin sulfate (specific activity, 1,842 dpm/μg) was a gift from G. Arcieri, Schering Corp., Bloomfield, N.J. An aqueous stock solution of 1,000 μg of free base per ml was prepared and used for the binding experiments within 6 months of storage at 4°C. Lysostaphin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in sterile isotonic saline at a concentration of 1,000 U/ml and kept at −40°C.

Highly purified trypsin (Sigma) in sterile isotonic saline at a concentration of 25 mg/ml, stored at −20°C, was used to inactivate lysostaphin in preliminary experiments. Subsequently, another trypsin preparation, showing a 1% contamination with chymotrypsin (Serva-Feinbiochemica, Heidelberg, Germany) was used and prepared freshly for each assay.

Ficoll and dextran T-70 and 500 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and Ronpacon was from Cilag-Chemie, Schaffhausen, Switzerland. A Dulbecco phosphate buffer saline solution (GIBCO-Bio-Cult Co., Glasgow, Scotland) was adjusted to pH 7.4 with NaOH and supplemented with 1/100 volume of a 10% sterile glucose solution. This supplemented buffer will be referred to as DG medium, or as DGS medium when enriched with 10% pooled human serum (stored at −80°C).

Dimilume 30 was purchased from Packard Instrument International S.A., Zürich, Switzerland. Oil Red-O (ORO), di-iso-dodecylphtalate, and *Escherichia coli* O26:B6 lipopolysaccharide were purchased from Allied Chemical Corp., Morristown, N.J. and Difco, respectively.

Gentamicin assays. The gentamicin concentrations were measured microbiologically, using a modified agar well method (4) and *Bacillus subtilis* ATCC 6633 as the test organism. Occasionally and when indicated, gentamicin concentrations were determined enzymatically, using partially purified gentamicin-adenine mononucleotide transferase (21).

Preparation of leukocytes. For the phagocytic bactericidal assays, leukocytes were purified by dextran sedimentation from venous blood of normal donors. Segmented PMN, representing usually 50 to 70% of the leukocyte preparation, were adjusted to a final concentration of 10^7/ml in DG medium, after enumeration in a hemacytometer.

Experiments recording the binding of radioactive gentamicin by PMN required extensive purification to minimize possible contamination and binding of the antibiotic by lymphomonocytes or erythrocytes; in this group of experiments, PMN were therefore purified by the Böyum technique (8). PMN constituted about 98% of the nucleated cells. Contamination by erythrocytes was reduced to 10 to 30% after three steps of hypotonic lysis in distilled water (3). Cell viability of the PMN was superior to 95% as estimated by the trypan blue dye exclusion test.

Phagocytic bactericidal assays. Conditions used for the phagocytic assay were as follows: each 1-ml incubation mixture included 5×10^6 PMN in DGS medium and 2×10^6 to 6×10^6 colony-forming units (CFU) of strain *S. aureus* Wood 46 (0.1 ml from a 50-fold diluted overnight culture in Mueller-Hinton broth). Incubations were performed at 37°C in siliconized tubes in a horizontal shaker at a rate of 240/min. The hypotonic lysis procedure of the PMN at the end of the incubation was found to be harmful to staphylococci in the presence of gentamicin, even in trace amounts. In all phagocytic bactericidal assays performed in this study, a modified lysis procedure of PMN, which preserved bacterial viability, was devised by diluting 0.1-ml portions of the phagocytic mixture in 10 ml of isotonic saline supplemented with 0.1% Triton X-100. In such a medium, lysis of PMN was achieved within 2 min without any significant killing of *S. aureus*. After further isotonic 10-fold dilution, if required, portions of the most appropriate dilution were plated on Mueller-Hinton agar, and residual CFU were determined after incubation at 37°C up to 48 h. When lysostaphin was included in the phagocytic assay, the antibiotic was inactivated before lysis of PMN by preincubation of each 0.1-ml portion for 10 min at 37°C with 0.9 ml of a 0.25% trypsin solution in saline (26); inactivation of lysostaphin by trypsin treatment under our experimental conditions was confirmed by negative residual antibiotic assays. Thereafter, the whole 1-ml portion was treated as described above.

To evaluate the effects of antibiotics on intracellular bacteria, ingestion had to be allowed to occur before addition of any antibacterial agent; this was achieved by a 10-min preincubation of opsonized bacteria and PMN. The bacterial killing rate of the system at 10 min was quantified by CFU counts as described above. After the 10 min of preincubation, either gentamicin or lysostaphin was added in 0.1-ml concentrated saline solutions. The kinetics of further bacterial killing were recorded by performing CFU counts at 10-min intervals. Each test was always run with controls lacking either the antimicrobial agent or the PMN.

Removal of one antimicrobial agent before addition of a second one was achieved by three centrifugations for 10 min at $300 \times g$, and washings in 0.1 ml of DGS of the suspension. PMN counts and viability studies showed an 80 to 100% recovery of the PMN, with more than 95% viability by the trypan blue exclusion test at the end of the procedure.

[^{14}C]gentamicin binding. Each 1-ml incubation mixture included 5×10^6 purified PMN and 100 μg (235,000 dpm) of [^{14}C]gentamicin in DGS medium with or without 9×10^6 CFU of *S. aureus* MR-GR 1 per ml, depending on whether antibiotic binding to phagocytosing or non-phagocytosing PMN was studied. Viability of *S. aureus* MR-GR 1 was unaffected by the high level of [^{14}C]gentamicin required for the binding experiments. Samples removed at 15 min and stained with methylene blue verified that almost complete ingestion of *S. aureus* had indeed taken place. In both experimental conditions, the cell suspensions were incubated for 60 min as described above. At 60 min, the incubation was stopped by chilling, and the PMN were thoroughly washed by three consecutive centrifugations at $300 \times g$ for 5 min and by suspension in 1 ml of ice-cold DGS medium supplemented with 100 μg of nonradioactive gentamicin per ml. Viability of PMN after each washing step was found to be >95%. After the third centrifugation, the PMN pellet was solubi-

lized in 0.5 ml of 0.8 N NaOH for 3 h, and portions of the dissolved material were mixed with 10 ml of an emulsion system selected for scintillation counting.

The contribution of [^{14}C]gentamicin binding to phagocytosed *S. aureus* was assessed by incubating 9 × 10^6 CFU of strain MR-GR 1 per ml in the absence of PMN with 100 μg of radioactive antibiotic per ml, in DGS medium for 60 min at 37°C. At 60 min, bacteria were washed, solubilized, and counted. This "piggy-back" binding of [^{14}C]gentamicin to actively phagocytosing PMN was minimized in another series of experiments by using opsonized, inert endotoxin-coated, ORO particles for ingestion, as described by Stossel (24). After 10 min of incubation in the presence of 100 μg of [^{14}C]gentamicin per ml, the PMN pellets were washed twice by suspension in 2 ml of fresh DGS medium supplemented with 100 μg of nonradioactive gentamicin. Viability of PMN after the wash was >95%, and >85% PMN contained ingested ORO particles. Determination of [^{14}C]gentamicin binding of the washed PMN was carried out after solubilization of the pellet in 1 ml of 0.8 N NaOH. Control experiments, performed with 0.2 ml of preopsonized ORO particles incubated with 100 μg of [^{14}C]gentamicin in 1 ml in DGS medium for 10 min at 37°C and harvested as a top layer after centrifugation at 3,000 × *g* for 10 min, showed that ORO particles did not bind any [^{14}C]-gentamicin significantly.

Radioactive counts. A 0.1-ml volume of the NaOH-solubilized PMN, calibrated with a high-precision pipette (Bie et Bernsten, Copenhagen, Denmark), was emulsified overnight in 10 ml of the scintillator Dimilume 30 and counted in a liquid scintillation counter (LS 3135-T, Beckman). The counting efficiency was 90%.

RESULTS

Bactericidal effect of gentamicin in the presence of PMN. Figure 1 demonstrates that the bactericidal effect of 5 μg of gentamicin per ml on *S. aureus* Wood 46 was markedly decreased in presence of phagocytosing PMN. When phagocytosis was allowed to proceed for 10 min before the antibiotic was added, CFU counts dropped by only 0.7 $\log_{10}$ during the following 50 min; in contrast, CFU counts dropped by 2.8 $\log_{10}$ after only 10 min of incubation when *S. aureus* Wood 46 was mixed with gentamicin in the absence of PMN, and further counts performed later were all inferior to 10^3 CFU/ml ($\Delta\log_{10} > 3.3$). An intermediate kill curve was obtained when PMN and gentamicin were added at the same time to *S. aureus*. In the presence of PMN, the addition of gentamicin at zero time resulted in a decrease in all counts performed between 20 and 60 min of about 1 $\log_{10}$, in comparison with the values obtained after 10 min of preincubation. Thus, not only the presence of PMN but also their early interaction with *S. aureus* was responsible for the inhibition of gentamicin antibacterial activity. Gentamicin itself was not inactivated by the phagocytic system since at the end of the 60-min

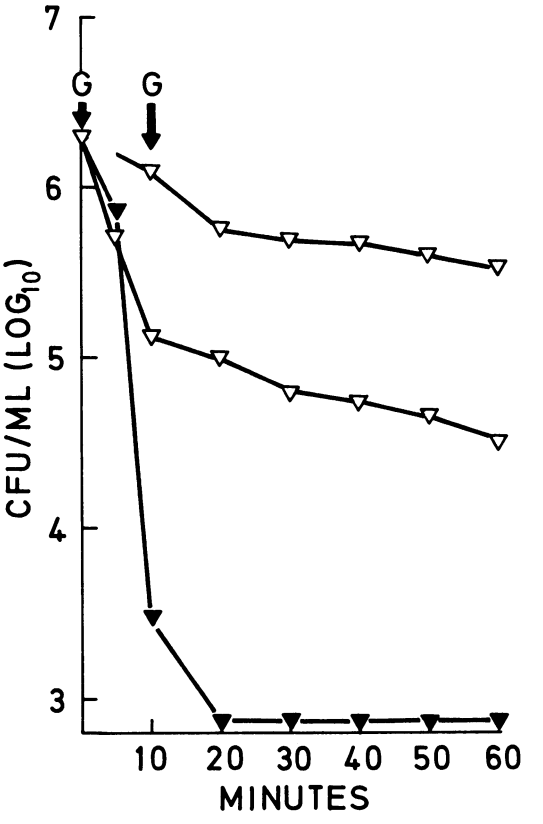

FIG. 1. *Time-kill curves of S. aureus Wood 46 by 5 μg of gentamicin (G) per ml in the absence (▼) or presence (▽) of phagocytosing 5 × 10^6 PMN/ml. In the experiments depicted by ▽, gentamicin was added either at time zero or after 10 min of incubation (arrows).*

period of incubation full activity of the antibiotic was recovered in the supernatant of the centrifuged PMN-bacteria mixture by either a microbiological (4) or an enzymatic (21) assay.

Since lysostaphin has been shown to be bactericidal for extraleukocytic *S. aureus* only (19, 26) in phagocytic assays, its effect was compared with that of gentamicin. Figure 2 shows that 5 μg of gentamicin per ml and 10 U of lysostaphin per ml, added separately to two phagocytic mixtures preincubated for 10 min with opsonized *S. aureus* and PMN, produced similar kill curves, the differences in CFU counts never exceeding 20% (n = 5). When tested in the absence of PMN, lysostaphin exerted a rapid killing effect on *S. aureus* which was complete at 5 min and reduced the CFU count by 1.3 to 1.7 $\log_{10}$.

These results taken together showed that both gentamicin and lysostaphin were less efficient bactericidal agents in the presence of phagocytosing PMN, but still raised the question of

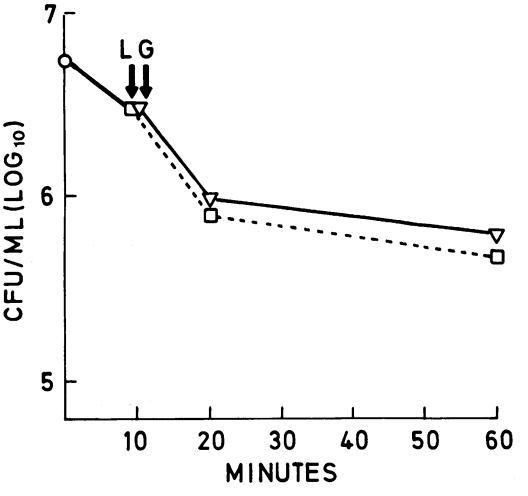

FIG. 2. *Time-kill curves of S. aureus by 5 μg of gentamicin per ml (▽) or by 10 U of lysostaphin per ml (□), added after 10 min of incubation with 5 × 10⁶ phagocytosing PMN/ml.*

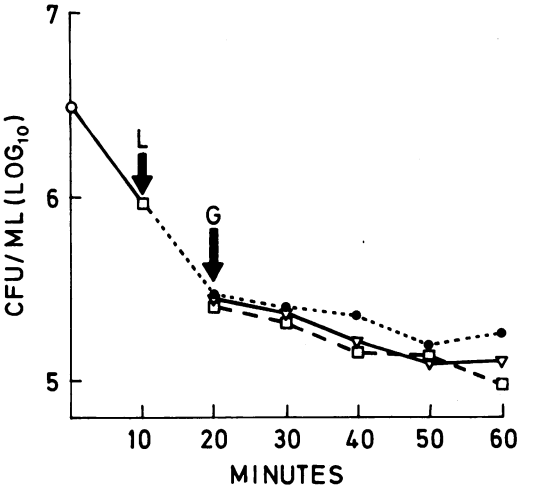

FIG. 3. *Time-kill curves of S. aureus by 5 (▽) or 50 (□) μg of gentamicin (G) per ml, or in the absence of the antibiotic (●). Before the addition of gentamicin (arrow G), S. aureus was phagocytosed by 5 × 10⁶ PMN/ml for 10 min, then extraleukocytic bacteria were destroyed by incubation with lysostaphin (arrow L) for 10 min.*

whether the poor antibacterial activity of gentamicin was indeed limited to intracellular organisms. This question was investigated by using the following experimental approach: PMN and *S. aureus* were incubated for 10 min as outlined previously, and lysostaphin was added at a concentration of 10 U/ml for a further 10 min. At 20 min, the incubation was divided into three portions. One received 5 μg of gentamicin per ml, the second received 50 μg of gentamicin per ml, and the last one received saline (control tube). Figure 3 demonstrates that the addition of gentamicin, at concentrations as high as 50 μg/ml, to a phagocytic mixture already treated with lysostaphin, did not modify the progressive decrease in CFU counts recorded in the control tube and resulting from the bactericidal activity of PMN. No modification could be recorded either after 150 or 300 min of incubation; in contrast, 0.6 μg of gentamicin per ml killed 3 $\log_{10}$ of *S. aureus* within 60 min in the absence of PMN (data not shown). In conclusion, gentamicin at 50 μg/ml was totally ineffective against residual *S. aureus* after the removal of extracellular organisms by lysostaphin in the presence of phagocytosing PMN. This lack of killing effect could not be ascribed to inactivation of the antibiotic, since activity of each antimicrobial agent was assayed at the end of these experiments and found to be unchanged. Lysostaphin having been shown to be without bactericidal effect on intracellular organisms, these results suggested that gentamicin-"insensitive," lysostaphin-"treated" residual microorganisms were the phagocytized intracellular staphylococci.

To rule out any inhibitory effect of lysostaphin on the gentamicin bactericidal effect, the same experiment as recorded in Fig. 3 was repeated, except that the phagocytic suspension was washed free of lysostaphin before receiving either gentamicin or saline (Fig. 4). At 20 min, the lysostaphin-treated phagocytic suspension was centrifuged three times and suspended in fresh DGS medium. During the washing steps, CFU counts decreased from 6.6 × 10^4 to 3.0 × 10^4 CFU/ml, whereas PMN counts and viability remained unchanged. After the last wash, one tube received 5 μg of gentamicin, another one received 50 μg/ml, and the last portion received only saline (control tube). In the absence of lysostaphin, gentamicin at 50 or 5 μg/ml was as inactive against lysostaphin-treated, PMN-associated bacteria as saline (Fig. 4).

Confirmation of the intraleukocytic location of the *S. aureus* surviving gentamicin treatment was attempted by the following experiments. After 60 min of incubation under experimental conditions identical to those depicted in Fig. 2 and 3, the phagocytic suspensions were removed and their PMN were lysed by the addition of 1/10 volume of a 1% Triton X-100 solution, allowing interaction of presumably intracellular, viable *S. aureus* with gentamicin. After 20 min of further incubation at 37°C, CFU counts of the

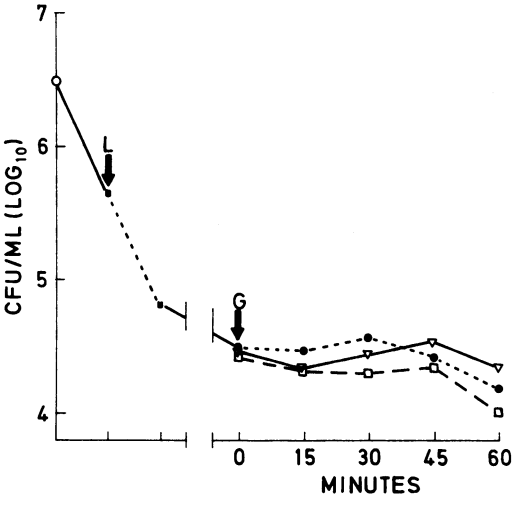

FIG. 4. *Time-kill curves of S. aureus by 5 (▽) or 50 (□) μg of gentamicin (G) per ml or in absence of the antibiotic (●). Phagocytosis and destruction of the extraleukocytic bacteria were performed exactly as described in the legend to Fig. 3, but the phagocytic suspension was washed free of lysostaphin before receiving the gentamicin.*

lysates including gentamicin were performed and shown to decrease by more than 2 $\log_{10}$.

Binding of [^{14}C]gentamicin by resting and phagocytosing purified PMN. Binding or uptake of gentamicin or both by PMN was determined by incubating highly purified preparations of PMN with 100 μg of [^{14}C]gentamicin per ml for 60 min at 37°C in DGS medium. Radioactive counts of [^{14}C]gentamicin bound to washed PMN were low, but reproducible and significantly higher ($P < 0.001$) than background values. Table 1 shows that 10^7 resting PMN bound 112 ± 10 ng ($n = 3$) of [^{14}C]gentamicin during the 60 min of incubation. When 10^7 PMN were actively involved in the phagocytosis and killing of 8.7×10^6 CFU of the gentamicin-resistant *S. aureus* strain MR-GR 1, they bound some additional [^{14}C]gentamicin compared to resting values. However, this nonsignificant increase in counts ($P > 0.05$) could be accounted for by the affinity of [^{14}C]gentamicin for *S. aureus* MR-GR 1, since 10^6 CFU of strain MR-GR 1 incubated for 60 min in DGS medium was shown to bind 92 ± 2 ng ($n = 4$) of [^{14}C]gentamicin. Similar results were obtained with heat-killed bacteria (Table 1).

Poor binding of [^{14}C]gentamicin to PMN involved in active phagocytosis was further demonstrated by the use of endotoxin-coated ORO particles, whose affinity for [^{14}C]gentamicin at 100 μg/ml was shown to be negligible. When 10^7 PMN were actively involved in ingestion of 0.5 mg of opsonized ORO particles for 10 min, they bound only 25% more [^{14}C]gentamicin than in the resting state (Table 1), and this increase was not significant ($P > 0.05$). Thus, [^{14}C]gentamicin binding by PMN was unaffected by the physiological state of the PMN, whether resting or involved in active phagocytosis.

The following observations suggested that the recorded binding values of [^{14}C]gentamicin to PMN reflected other processes than active uptake of the antibiotic by the leukocytes. (i) When 10^7 purified, resting PMN were incubated with 100 μg of [^{14}C]gentamicin per ml as described above, but washed thereafter with a higher concentration (1 mg/ml) of nonradioactive gentamicin in DGS medium, there was a tendency for less [^{14}C]gentamicin binding (66 ± 17 ng, $n = 3$) than under control conditions (151 ± 65, $n = 3$), i.e., 100 μg of nonradioactive gentamicin in DGS medium ($P > 0.05$). Thus a high concentration of nonradioactive gentamicin added to the washing medium seemed to elute some of the [^{14}C]-gentamicin molecules presumably adsorbed at the surface of the PMN. (ii) Very high binding values of [^{14}C]gentamicin in the presence of PMN were occasionally recorded in replicate assays. Exceptionally high values of 637 and 6,522 ng of [^{14}C]gentamicin per 10^7 PMN were

TABLE 1. *Comparison of binding of [^{14}C]-gentamicin to resting or actively phagocytosing human PMN*[a]

PMN	Particles ingested	Binding (ng/10^7 PMN)
Present	None	112 ± 10 (3)[b]
Present	*S. aureus* alive[c]	193 ± 40 (4)[d]
Present	*S. aureus* heat-killed[c]	150 ± 18 (4)[d]
Absent	*S. aureus* alive	92 ± 2 (4)[e]
Absent	*S. aureus* heat-killed	82 ± 3 (4)[e]
Present	ORO particles (0.5 mg)[f]	140 ± 35 (3)[d]

[a] If not specified, incubation was performed for 60 min at 37°C with 100 μg of [^{14}C]gentamicin per ml.

[b] Values represent means ± standard error of the mean. Numbers in parentheses indicate number of determinations.

[c] 8.7×10^6 *S. aureus* cells/ml.

[d] *P* values, determined by unpaired Student's *t* test comparing values for resting PMN to actively phagocytosing PMN, are >0.05.

[e] Binding is expressed in nanograms per 10^6 cells of *S. aureus*.

[f] 10-min incubation; 0.5 mg of ORO corresponds to 10^8 particles.

recorded once each, the other replicate assays of the same experiment yielding lower binding values ranging from 43 to 277 ng. These occasionally high binding values were suspected to be due to partial lysis of the PMN. Experiments performed with entirely lysed preparations of purified PMN added to intact PMN suspensions showed indeed that a 1% contamination with lysed material of a purified PMN suspension containing 10^7 cells/ml could result in the binding of 100 ng of [^{14}C]gentamicin.

DISCUSSION

Attempts made by previous authors to ascertain the intraleukocytic effect of antibiotics on phagocytized bacteria have been hampered by several technical difficulties. In particular, the problem of the determination of the exact number of intraleukocytic viable bacteria present at the time of antibiotic addition has not been adequately solved (1, 10, 13, 14, 20, 22, 23). Thus, no information regarding the initial penetration of antibiotics added to the phagocytic assay could be obtained, because the initial decrease in CFU reflected in part the elimination of the residual extraleukocytic bacteria by the antibiotic (1, 10, 13, 14, 20, 22, 23). Ideally, such a system requires rapid and almost complete ingestion of the test bacteria, elimination of extracellular, noningested microorganisms, rapid killing effect of the antibiotic tested in a cell-free system, and delayed bactericidal contribution of the intracellular, PMN-dependent killing mechanisms. Our approach to evaluating the initial bactericidal effect of gentamicin on intraleukocytic *S. aureus* consisted of incubating the antibiotic with a phagocytic suspension after extensive ingestion followed by lysostaphin treatment, in such a way that CFU counts reflected mainly the viability of intraleukocytic bacteria. Previous studies from our laboratory have confirmed the findings of Björksten et al. (5) that ingestion of *S. aureus* in a PMN phagocytic system is extensive after 10 min. Lysis of all extracellular *S. aureus* by lysostaphin has been repeatedly demonstrated (5, 19, 26).

Our present study shows that gentamicin added at a highly bactericidal concentration to preingested *S. aureus* exerts no killing effect on the population of intraleukocytic microorganisms. This inhibition of gentamicin killing effect was a long-lasting phenomenon, since no delayed killing occurred after several hours of incubation. Two parameters had to be checked carefully during these experiments. First, viability of the PMN was confirmed to remain constant for several hours of incubation, as checked by the dye exclusion test, and it was unaffected by the presence or absence of lysostaphin or gentamicin or both. Furthermore, functional integrity of the PMN after several hours of incubation under similar experimental conditions has been documented by rechallenge experiments (27). Second, it had to be demonstrated that extraleukocytic bactericidal activity of gentamicin remained constant throughout the experiments and was unaffected by PMN, since recent observations have suggested inactivation of aminoglycosidic antibiotics by inflammatory exudates (7). The following observations suggest that gentamicin antibacterial activity was intact during the assay. (i) Full activity of the antibiotic was recovered at the end of the incubation period, as assayed by standard microbiological or enzymatic procedures (4, 21). (ii) The impeded bactericidal activity of gentamicin in presence of a PMN suspension was restored after Triton X-100 lysis of the PMN, bringing the formerly intraleukocytic *S. aureus* into contact with the antibiotic. A possible interfering effect of lysostaphin on gentamicin bactericidal activity against intraleukocytic *S. aureus* was also excluded by appropriate control experiments, since gentamicin was still unable to kill intraleukocytic *S. aureus* after lysostaphin had been eliminated from the phagocytic suspension. Also, lysostaphin-treated *S. aureus* were still susceptible to added gentamicin (data not shown) in a cell-free system.

Evaluation of gentamicin binding by PMN, whether resting or involved in active phagocytosis, was complicated by the high affinity of [^{14}C]gentamicin for unknown substrates resulting from PMN lysis. Traces (1%) of this material purposely contaminating the purified preparation of intact PMN increased significantly the radioactive counts of cell-associated [^{14}C]gentamicin. This contribution was therefore difficult to quantify in our experiments, since a 1% lysis of PMN was undetectable by the dye exclusion test. The fact that high counts (10- to 100-fold) were recorded in a few assays only, whereas most showed low cell-associated radioactive counts, favors the interpretation that this artifact occurred only occasionally in our system. Further experiments (manuscript in preparation) suggest that this binding occurs on nuclear material. Even with this restriction, the maximal amount of radioactive gentamicin which entered the PMN can be estimated to be low; 10^7 PMN contain approximately 2 μl of intracellular water (14), and 43 ng of gentamicin distributed in this volume could result in a final intracellular concentration of 21.5 μg/ml. This estimation is well below the 100-μg/ml extraleukocytic concentration of [^{14}C]gentamicin used in our phagocytic system.

Our results allow us, therefore, to suggest that

gentamicin does not accumulate in the intracellular space of PMN and that its intracellular concentration is even lower than that expected from simple diffusion mechanisms. Poor diffusibility of charged molecules across eukaryotic cell membranes has been amply documented (18), and such a principle seems to be valid for gentamicin also.

In conclusion, the inability of gentamicin to kill intraleukocytic *S. aureus* is due partly to its poor penetration into phagocytes. It is also conceivable that the low pH prevailing in the phagosome reduces the bactericidal activity of gentamicin as documented in a cell-free system (2). Finally, intraleukocytic *S. aureus* could become insensitive to gentamicin by a leukocyte-induced defect in its aerobic active transport mechanism (6), thus inhibiting the accumulation of bactericidal amounts of this antibiotic. All these factors acting together can contribute to the protection of intraleukocytic bacteria from gentamicin bactericidal action, as demonstrated in our study.

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