Influence of Subinhibitory Concentrations of Penicillin, Cephalothin, and Clindamycin on \textit{Staphylococcus aureus} Growth in Human Phagocytic Cells

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After an initial 2-h incubation with phagocytic cells, the growth of surviving intracellular \textit{Staphylococcus aureus} was examined in the presence of subinhibitory concentrations of penicillin, cephalothin, and clindamycin. One-tenth of the minimal inhibitory concentrations of these antibiotics markedly reduced bacterial growth in normal polymorphonuclear leukocytes. In contrast, when human alveolar macrophages were studied, no inhibition of growth was seen. Subinhibitory concentrations of these antibiotics and polymorphonuclear leukocytes acted synergistically to reduce intracellular survival of \textit{S. aureus}. This synergy did not appear to be dependent upon the microbicidal potential of the leukocyte respiratory burst, since no differences were found when polymorphonuclear leukocytes obtained from patients with chronic granulomatous disease were compared with those from normal donors.

Antibiotic penetration of phagocytic cells may be a critical factor in the fate of intracellular bacteria. It is reported that phagocytized bacteria are protected from the effect of antibiotics (5, 10, 19, 21); however, enhancement of the bactericidal capacity of polymorphonuclear leukocytes (PMN) may be secondary to the effect of antibiotic-exposed bacteria before phagocytosis. Even at subinhibitory concentrations of antibiotics, bacterial morphology (2, 9, 18), adherence properties (15), opsonic requirements (2), and kinetics of phagocytosis and killing by PMN are altered (1, 2, 6, 9, 11, 17, 18).

In this investigation, we evaluated the effects of subinhibitory concentrations of penicillin, cephalothin, and clindamycin on the intracellular multiplication of \textit{Staphylococcus aureus} in PMN obtained from healthy donors and from patients with chronic granulomatous disease (CGD) and in human alveolar macrophages (AMΦ). Despite different capacities to penetrate phagocytic cells, these antibiotics acted synergistically with PMN to reduce the intracellular survival of \textit{S. aureus}.

MATERIALS AND METHODS

Preparation of bacteria. \textit{S. aureus} Cowan I stock culture was inoculated in 10 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) and placed in a 37°C shaker incubator for 18 h. The bacteria were harvested, washed three times with phosphate-buffered saline (pH 7.4), and then adjusted to $5 \times 10^8$ colony-forming units (CFU) per ml in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.).

\textbf{Antimicrobial agents.} Cephalothin, cephalothin, and penicillin G standard powders were concentrated in 10-mg/ml stock solutions by the method of Washington and Barry (23). Clindamycin was supplied by The Upjohn Co., Kalamazoo, Mich., cephalothin was supplied by Eli Lilly & Co., Indianapolis, Ind., and penicillin G (PEN-NA) was supplied by Sigma Chemical Co., St. Louis, Mo. Minimal inhibitory concentrations (MICs) were determined by a standard broth dilution technique (14). When RPMI 1640 medium was used in the MIC determinations, the endpoints were either the same or varied by less than one dilution. The MICs for \textit{S. aureus} used in this report were 0.5 μg/ml for clindamycin and cephalothin and 0.1 μg/ml for penicillin G.

Preparation of PMN. Heparinized venous blood (10 U of heparin per ml of blood) was obtained from healthy adults and from uninfected patients with CGD. Whole blood was settled by gravity with 6% dextran in normal saline (3 ml of dextran per 10 ml of blood), and the PMN-rich supernatant was layered on Lymphocyte Separation Medium (Litton Bionetics, Kensington, Md.). After centrifugation for 30 min at $300 \times g$, the pellet was removed, the erythrocytes were lysed with 0.87% NH₄Cl, and the remaining PMN were washed three times in Hanks balanced salt solution containing 0.1% gelatin. Final volumes were adjusted to $5 \times 10^8$ PMN per ml.

Preparation of AMΦ. AMΦ were obtained from healthy adult volunteers by segmental lavage with sterile normal saline with a fiberoptic bronchoscope (4). AMΦ were separated by centrifugation at $200 \times g$ for 10 min, washed three times with Hanks balanced salt solution containing 0.1% gelatin, and suspended to $5 \times 10^6$ cells per ml. AMΦ suspensions were greater
This number of CFU was used as the zero time for the initiation of intracellular growth. The postphagocytic mixture was then divided into 0.2-ml samples in a 24-well tissue culture plate (Costar, Cambridge, Mass.), and 0.2 ml of RPMI 1640 medium was added to each well. Antibiotics were added to the tissue culture medium in concentrations of 1/10 or 1/40 the MIC. After 16 h, the cells were lysed with sterile water, serially diluted, and the number of CFU was determined. Most assays were done three times each with triplicate samples. The number of CFU present at the zero postphagocytic time was compared with the number of CFU after the 16-h time to quantify growth. Cells were also examined microscopically at 10 and 16 h and compared with cells at the zero time for morphological evaluation of intracellular bacterial growth.

**Viability and chemiluminescence assays.** Viability of phagocytes at various times over the 16-h incubation period was determined by trypan blue exclusion. In addition, cells were evaluated for their ability to elicit a chemiluminescence response to opsonized zymosan at the various times, using a luminol-enhanced chemiluminescence assay (12).

**RESULTS**

Two hours after adding $2.5 \times 10^9$ CFU/ml to the phagocytic mixtures, intracellular survival of *S. aureus* was $2.8 \times 10^4$, $1.45 \times 10^5$, and $4 \times 10^5$ CFU/ml for normal PMN, PMN from patients with CGD, and AMΦ, respectively. After 16 h of incubation, there was a 2- to 3-log increase in the number of CFU with each cell type (Fig. 1). When 1/10 the MIC of penicillin, cephalothin, or clindamycin was added to the phagocytic mixture containing normal PMN, bacterial growth was completely inhibited. When 1/40 the MIC of these antibiotics was added, an intermediate increase of intracellular bacteria was observed; however, this increase was significantly less than that observed with the control.

Figure 2A through C shows representative changes observed microscopically in PMN after incubation with *S. aureus* for the zero, 10-, and 16-h times in the control wells. More intracellular *S. aureus* cells were visible at 10 h than at the zero time, and after 16 h, both intracellular and extracellular bacteria were observed. Figure 2D shows the absence of intracellular growth of bacteria when incubated in the presence of 1/10 the MIC of clindamycin.

When *S. aureus* were incubated with PMN from patients with CGD, there was a higher number of CFU at zero time (Fig. 1B) as compared with the number observed with normal PMN. However, intracellular survival of *S. aureus* in the presence of sub-MICs of penicillin, cephalothin, and clindamycin was similar in PMN from patients with CGD and normal PMN.

The bactericidal capacity of AMΦ was similar to that of PMN from patients with CGD, with $4 \times 10^5$ intracellular *S. aureus* per ml present at the zero time (Fig. 1C). In contrast to normal
PMN and PMN from patients with CGD, there was no significant difference in intracellular numbers of intracellular 
*Staphylococcus aureus* in AMΦ with or without cephalothin and clindamycin. There were reduced numbers of intracellular 
*S. aureus* in the presence of 1/10 the MIC of penicillin (experiment performed with AMΦ of only one donor).

The PMN were 88, 87, 74, and 64% viable as measured by trypan blue exclusion at the zero, 2-, 10-, and 16-h times, respectively. The AMΦ were 90, 74, 68, and 50% viable for the same times, respectively. When peak chemiluminescence of the PMN in our test system was compared with that of normal freshly isolated PMN, we found values of 20 and 17% that obtained from normal PMN at the zero and 2-h times, respectively.

**DISCUSSION**

Although *S. aureus* are rapidly killed by normal phagocytic cells, a small percentage of ingested staphylococci survive and are believed to be of major importance in some staphylococcal infections (5, 13). Sub-MICs of clindamycin and two β-lactam antibiotics, penicillin and cephalothin, prevented intracellular survival within normal PMN. One important mechanism whereby PMN kill staphylococci is by the generation of reactive oxygen species. The PMN in our assay were still capable of producing a chemiluminescence response after phagocytosis of *S. aureus* for 2 h. However, the inhibition of growth in PMN from patients with CGD was similar, suggesting that the synergistic effect of sub-MICs of antibiotics and PMN does not depend on the formation of reactive oxygen species.

It has recently been demonstrated that the concentration of clindamycin within normal PMN is up to 40 times higher than that in the extracellular milieu (8, 16). Our findings may be explained by this property of clindamycin; however, sub-MICs of penicillin and cephalothin also prevented intracellular survival of *S. aureus*. Most studies of antibiotic penetration into phagocytic cells have not been carried out with cells which had already participated in phagocytosis. It seems possible that penicillin and cephalothin may penetrate PMN because the PMN had phagocytized *S. aureus* before incubation with antibiotics. Veal et al. (22) showed that penicillin penetrates PMN containing gonococci. Root et al. (18) have shown that *S. aureus* killing was enhanced by pretreatment with sub-MICs of penicillin before phagocytosis. Since low concentrations of these antibiotics may enter cells (16), they may alter the bacterial cell wall, rendering *S. aureus* more susceptible to PMN bactericidal activity.

Other investigators (3, 7) have demonstrated that clindamycin is actively concentrated by rabbit AMΦ; however, they did not find an antimicrobial effect on intracellular staphylococci (7). In the present study, we used human AMΦ from healthy donors and were similarly unable to find an inhibitory effect of sub-MICs of clindamycin on intracellular growth of *S. aureus*. Cephalothin was also unable to significantly limit the growth of *S. aureus* that had been ingested by human AMΦ; however, in one test, penicillin did inhibit growth somewhat. These findings suggest that an oxygen-independent antimicrobial system of PMN is responsible for the killing of staphylococci in the presence of sub-MICs of antibiotics in PMN and that these factors are either not operative or not optimally effective in human AMΦ.

Antibiotics may be clinically effective even if infecting bacteria are insensitive by standard in vitro sensitivity testing. The results of this study suggest that this laboratory-clinical paradox may be explained by the antibacterial activity of antibiotics at concentrations well below the in vitro inhibitory concentrations in the presence of PMN.

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LITERATURE CITED


