Effect of Zinc-Reversible Growth-Inhibitory Activity in Human Empyema Fluid on Antibiotic Microbicidal Activity

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Abscess fluid supernatants have zinc-reversible microbial growth-inhibitory activity that is mediated by calprotectin, a zinc-binding protein. Because it inhibits microbial growth, this activity might interfere with killing by antibiotics that require their target organisms to be proliferating. In the present study, we cultured bacteria in human empyema fluid and used zinc to overcome the growth-inhibitory effect of calprotectin. We then compared the effect of zinc on killing by the beta-lactams ampicillin and cefazolin with that of the fluoroquinolone trovafloxacin, since the latter may be better able to kill nonproliferating organisms. In empyema fluid diluted 1:5 in normal saline, addition of zinc (30 μM) increased growth of two strains of Staphylococcus aureus and two strains of Escherichia coli but did not affect the MICs or MBCs of the three antibiotics in Mueller-Hinton broth. For one strain of S. aureus, no effect of zinc was found on killing by either ampicillin or cefazolin. However, with the other strain of S. aureus and both strains of E. coli, significant enhancement of killing by both drugs was observed with zinc addition. On the other hand, no effect on the killing of any of the organisms was observed for trovafloxacin when zinc was added. These results suggest that the zinc-reversible growth-inhibitory activity of abscess fluid may interfere with the microbicidal activity of antibiotics requiring proliferating target organisms, although antibiotics better able to kill nonproliferating organisms may be less affected by this phenomenon.

Microorganisms require metals, such as iron and zinc, for growth. Sequestration of these metal ions by host metal-binding proteins can be an effective means of antimicrobial defense. This mechanism has been described primarily as a system involving host iron-binding proteins, such as lactoferrin and transferrin (7, 25). However, a similar mechanism that is based on zinc rather than iron has recently been described (17). The substance responsible for this antimicrobial effect is the calcium- and zinc-binding protein complex called calprotectin; alternative names for this complex include the L1 protein, MRP 8 and MRP 14, calgranulin A and B, and the cystic fibrosis antigen (6). Calprotectin appears to originate in the cytoplasm of neutrophils and is then released at sites of infection as these cells die and lyse; this protein has microbistatic activity against a variety of bacterial and fungal microorganisms (13, 18, 21). Abscess fluid supernatants have been shown to contain large amounts of calprotectin and to possess similar antimicrobial activity in both the logarithmic and stationary phases of microbial growth, at least against certain microorganisms (26). There is also some evidence to suggest that this class of antibiotics may be relatively more effective in killing microorganisms in abscesses (4). The present study was undertaken to confirm the effects of calprotectin on microbial killing by beta-lactam antibiotics and to determine whether or not the fluoroquinolone antibiotic trovafloxacin is similarly affected.

**MATERIALS AND METHODS**

**Calprotectin-containing fluids.** A specimen of human pleural empyema fluid obtained before the patient had been treated with antibiotics was used for these studies. The fluid was centrifuged at 1,500 × g for 30 min before further use. Protein concentration was determined by a dye-binding assay (Bio-Rad Laboratories, Richmond, Calif.) to be 143 mg/ml for the undiluted fluid.

**Antimicrobial agents.** These studies used ampicillin and cefazolin from Sigma (St. Louis, Mo.) and trovafloxacin from Pfizer. The compounds were solubilized and diluted according to standard procedures. Fresh solutions were made for each experiment.

**Microorganisms.** We used four microbial strains for most of the studies. These included ATCC isolates 29213 and 25923 of S. aureus and ATCC isolate 25923 and a local clinical isolate (LC3) of Escherichia coli.

**MICs and MBCs.** We carried out microtiter plate MIC and minimal bactericidal concentration (MBC) determinations with a modification of standard methods (9). Doubling dilutions of the different antibiotics, from 0.01 to 32 μg/ml in 0.1-ml volumes of Mueller-Hinton broth, were tested with inocula of either 10² or 10⁵ organisms per ml. Since we previously found the effect of calprotectin to...
TABLE 1. Effect of zinc on antibiotic MBCs for E. coli and S. aureus in Mueller-Hinton broth

<table>
<thead>
<tr>
<th>Antibiotic and organism</th>
<th>MBCs (μg/ml) of antibiotic alone</th>
<th>MBCs (μg/ml) of antibiotic plus 30 μM zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus 29213</td>
<td>0.25–1 (0.5)</td>
<td>0.25–0.5 (0.5)</td>
</tr>
<tr>
<td>S. aureus 25923</td>
<td>0.5–4 (0.5)</td>
<td>1–8 (4)</td>
</tr>
<tr>
<td>E. coli 25922</td>
<td>1–4 (4)</td>
<td>0.5–4 (4)</td>
</tr>
<tr>
<td>E. coli LCI</td>
<td>2–2 (2)</td>
<td>2–2 (2)</td>
</tr>
<tr>
<td>Cefazolin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus 29213</td>
<td>0.5–1 (1)</td>
<td>1–4 (2)</td>
</tr>
<tr>
<td>S. aureus 25923</td>
<td>1–4 (2)</td>
<td>0.5–2 (2)</td>
</tr>
<tr>
<td>E. coli 25922</td>
<td>1–2 (2)</td>
<td>0.25–2 (2)</td>
</tr>
<tr>
<td>E. coli LCI</td>
<td>0.5–2 (1)</td>
<td>0.25–1 (1)</td>
</tr>
<tr>
<td>Trovafloxacin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus 29213</td>
<td>0.03–0.06 (0.03)</td>
<td>0.03–0.06 (0.06)</td>
</tr>
<tr>
<td>S. aureus 25923</td>
<td>0.03–0.1 (0.03)</td>
<td>0.03–0.06 (0.06)</td>
</tr>
<tr>
<td>E. coli 25922</td>
<td>0.01–0.03 (0.01)</td>
<td>0.01–0.03 (0.03)</td>
</tr>
<tr>
<td>E. coli LCI</td>
<td>0.01–0.03 (0.01)</td>
<td>0.01–0.03 (0.03)</td>
</tr>
</tbody>
</table>

* Data represent range and median of MBC data from three experiments per point for each isolate in broth alone or in broth containing 30 μM ZnSO₄. Differences between control and zinc-containing samples were evaluated with mean values in the unpaired t test, and none were found to be significant. Similar results were obtained with MIC determinations (data not shown).

The effect of zinc on antibiotic microbicidal activity in abscess fluid was tested with organisms grown directly in human empyema fluid diluted 1:5 in saline (final total protein concentration, 28.6 mg/ml). Different antibiotic concentrations were tested, with results from the concentration yielding a number of CFU closest to the original inoculum after incubation being compared for samples with and without added zinc. In the absence of antibiotic, zinc produced increased growth of the organisms in empyema fluid, although the amount of growth stimulation varied with the bacterial isolates (Table 2). With one isolate of S. aureus, 29213, such increases were found for samples containing each of the three antibiotics, as shown in Table 2. However, for the second isolate of S. aureus and the two isolates of E. coli, the numbers of CFU remaining in the samples with either cefazolin or ampicillin were significantly decreased when zinc had been added to the samples, as is also shown in Table 2. Presumably, the increase in growth caused by zinc addition made the organisms more susceptible to the microbicidal activity of the two beta-lactam antibiotics. On the other hand, addition of zinc did not significantly decrease the number of organisms remaining in the trovafloxacin-con-
taining samples for any of the isolates tested, as is also shown in Table 2. There did not appear to be a clear relationship between the stimulation of growth and the resulting enhance-
ment of antibiotic microbicidal activity; the isolate yielding the highest stimulation with zinc was the _S. aureus_ isolate that did not show the increased killing effect when zinc was added.

**DISCUSSION**

In these studies, addition of zinc increased bacterial growth in human empyema fluid, an effect that has previously been related to overcoming the zinc-binding antimicrobial activity of calprotectin in such fluid (19, 20). On the other hand, in the present study addition of zinc did not affect the MICs or MBCs of the three antibiotics in Mueller-Hinton broth. For one strain of _S. aureus_, no reductions of CFU were observed for any of the antibiotics when zinc was added; however, with the other isolate of _S. aureus_ and the two isolates of _E. coli_, addition of zinc significantly decreased the number of CFU present in the cefazolin- and ampicillin-containing samples. In contrast, no significant reductions in CFU of any of the organisms were observed for samples containing trovafloxacin when zinc was added. Therefore, under the conditions of these experiments, stimulation of bacterial growth by addition of zinc appeared to enhance the killing ability of the two beta-lactam antibiotics, but not that of the fluoroquinolone, trovafloxacin.

Antibiotics are known to work poorly in abscesses. Often this type of infection will persist for long periods unless the abscesses are drained (14). Neutrophils appear to localize less well to chronic abscesses than to acute ones (1). The presence of abscesses appears to inhibit the bactericidal activity of blood neutrophils, and the abscess fluid milieu is inhibitory to neutrophil function (2). In addition, it has been found that there is a poorly responsive subpopulation of neutrophils in abscess cell populations and that these cells contain higher numbers of abscess-derived bacteria (10). There are a variety of substances and conditions in abscess fluids that may interfere with the activity of various antibiotics, including beta-lactamases, DNA, and acidic pH (4). It is also likely that the nondividing state of the infecting microorganisms in abscesses may be at least partly at fault. This effect of the abscess fluid milieu may be the result of an active process involving sequestration of zinc by the neutrophil protein calprotectin. It is possible that other factors relevant to chronic infections may prevent the organisms from proliferating, but as demonstrated by Bamberger et al. (3) and confirmed by the present studies, addition of zinc to abscess fluid supernatants will stimulate bacterial growth in them.

Beta-lactam antibiotics are definitely better at killing proliferating organisms than nonproliferating ones. For example, although group A streptococci are known to be exquisitely sensitive to penicillin, this drug is sometimes relatively ineffective in treating infections caused by them (23). Stevens et al. have demonstrated better results with drugs like clindamycin and erythromycin that do not depend as much on growth for their killing activity, such as the fluoroquinolone, trovafloxacin. This phenomenon appeared to be isolate and concentration dependent. Thus, zinc may enhance microbicidal activity by suppressing the microbicidal effects of the beta-lactams than the total number of organisms generated during the entire incubation period.

In summary, an effect was found whereby zinc stimulation of bacterial growth in human empyema fluid enhanced the ability of ampicillin and cefazolin to kill the growing organisms, whereas the same was not true for the fluoroquinolone, trovafloxacin. This phenomenon appeared to be isolate and concentration dependent. Thus, zinc may enhance microbicidal activity by suppressing the microbicidal effect of the neutrophil protein calprotectin and increasing growth. Antibiotics that do not depend as much on growth for their killing activity, such as the fluoroquinolone, trovafloxacin, apparently are not subject to this effect. It is possible that with certain bacterial isolates and antibiotic concentrations, drugs of the fluoroquinolone class may show better microbicidal activity in abscess fluids than do beta-lactams.

**ACKNOWLEDGMENTS**

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**REFERENCES**

