Interaction of Cefotaxime and Aminoglycosides Against Enterococci In Vitro

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Enterococci have emerged as important bacterial pathogens that are frequently resistant to the new cephalosporin antimicrobial agents. Because these new agents are often utilized in combination with aminoglycosides, we thought it would be interesting to determine the in vitro activity of combinations of cefotaxime, the first third-generation agent available in the United States, and the three most commonly used aminoglycosides, gentamicin, tobramycin, and amikacin, to determine whether cefotaxime plus these agents might be reasonable candidates for use in patients with enterococcal disease.

MATERIALS AND METHODS

Fifty strains of enterococci were obtained from 24 urine cultures, 20 wound cultures, 2 sputum cultures, 2 bronchial washings, and 2 bile cultures performed in the Clinical Microbiology Laboratory at the University of Alabama Medical Center during November and December 1982. All strains grew in 6.5% NaCl heart infusion broth and as brownish-black colonies surrounded by a black zone on bile esculin agar. Precise determination of species was performed by the API 20ST Streptococcus System (Analytab Products, Plainview, N.Y.). There were 46 strains of Streptococcus faecalis and 4 strains of Streptococcus faecium.

Antimicrobial agents used in this study were kindly supplied as sterile standard reference powders by the following companies: cefotaxime by Hoechst-Roussell Pharmaceuticals, Inc., Somerville, N.J.; gentamicin by Schering Corp., Kenilworth, N.J.; tobramycin by Eli Lilly & Co., Indianapolis, Ind.; and amikacin by Bristol Laboratories, Syracuse, N.Y.

The minimal inhibitory concentration (MIC) was determined for each isolate for cefotaxime, gentamicin, tobramycin, and amikacin by both agar dilution and broth dilution techniques. In the broth dilution technique, five colonies of each strain were picked to 2 ml of Mueller-Hinton broth (BBL Microbiology Systems, Cockeysville, Md.), which were incubated overnight at 37°C and then diluted to a final inoculum of 5 x 10^3 CFU/ml. Serial twofold dilutions were made in Mueller-Hinton broth, using borosilicate tubes with loose-fitting metal caps with a total volume of 1 ml. The MIC was defined as the lowest concentration of the antimicrobial agent causing no visible growth after incubation at 37°C for 18 h.

From each clear tube and the cloudy tube with the least antimicrobial concentration, 0.01 ml was subcultured onto 5% sheep blood agar. The minimal bactericidal concentration (MBC) was defined as the lowest concentration showing five or less colonies after overnight incubation, representing 99.9% kill (1).

MICs were also determined by the agar dilution method described by Thornsberry et al. (6). To prepare agar dilution plates, 1 volume of each solution of antimicrobial agent was added to 9 volumes of unsupplemented molten Mueller-Hinton agar which was allowed to equilibrate in a water bath at 48°C and poured into petri dishes (100 by 15 mm). The following concentrations were utilized: cefotaxime, gentamicin, and amikacin, 2 to 2,048 µg/ml; tobramycin, 2 to 64 µg/ml (because the reference standard was obtained in a solution of 1,000 µg/ml, and 64 µg/ml was the highest concentration that could be studied after appropriate dilutions). The inoculum was prepared by touching five colonies of each strain and transferring them to 5 ml of Mueller-Hinton broth. Tubes were incubated at 37°C until visible turbidity was detected and then adjusted to a 0.5 McFarland standard. The suspensions were then diluted 1:10 in sterile broth so that the final concentration was 10^7 CFU/ml. Plates were inoculated with a Steers replicator (Melrose Machine Shop, Woodlyn, Pa.), resulting in a final inoculum of 10^9 CFU. Plates were incubated 16 to 20 h, with the MIC defined as the lowest concentration of antimicrobial agent at which complete inhibition occurred.

Tests for synergy were performed in Mueller-Hinton broth by the time-kill method. Sterile stock solutions were prepared from standard reference powders and...
further diluted to the approximate concentrations available in serum or concentrations one-fourth the MBC, whichever was lower. Concentrations employed were: cefotaxime, 100 µg/ml; gentamicin, 0.3 to 5.0 µg/ml, depending upon the particular strain; amikacin, 20 µg/ml; tobramycin, 5 µg/ml. Combinations were made of the following: cefotaxime, 100 µg/ml, with gentamicin, 0.3 to 5.0 µg/ml, depending on the strain; cefotaxime, 100 µg/ml, with amikacin, 20 µg/ml; cefotaxime, 100 µg/ml, with tobramycin, 5 µg/ml.

Overnight cultures of enterococci were diluted 1:10 to a concentration of 10^5 CFU/ml. From this dilution, 0.5 ml was added to 9.5-ml volumes of the concentrations of antibiotics in Mueller-Hinton broth described above as well as in Mueller-Hinton broth alone, which served as a control. This resulted in a final bacterial concentration of 5 x 10^5 CFU/ml (range, 9 x 10^5 to 8 x 10^5 as determined by serial dilutions and colony counts).

Tubes were incubated at 37°C, and at 0, 4, and 20 h viable colonies were enumerated by using a platinum loop calibrated to deliver 0.001 ml. Care was taken to insert the loop vertically into the test culture just below the surface, avoiding transfer on the wire above the loop. This loopful of broth was then seeded into melted nutrient agar that was cooled to 45°C, mixed, and poured into a petri dish (100 by 15 mm). After overnight incubation or until visible growth occurred on the growth control plate, the total number of CFUs were determined by using a magnifying glass counter (Quebec Colony Counter, American Optical Corp., Buffalo, N.Y.). If no growth occurred from the tubes containing antimicrobial agents, the time-kil test was repeated, increasing the sample subcultured to 0.1 ml. Colony counts were determined in parallel with 10 strains by adding 0.5 ml of the sample to 4.5-ml tubes of sterile saline (0.9% NaCl), and 10-fold serial dilutions were performed. A 0.025-ml portion was removed from each tube with a calibrated dropper (Microtiter micropipet; Dynatech Laboratories, Inc., Alexandria, Va.) and pipetted onto a 5% sheep blood agar plate that had been dried for 60 min at 37°C. Plates were incubated overnight or until the growth control became positive, and colonies were counted (3).

A paired t test was performed and showed no significant difference between the two methods (data not shown). Therefore, the calibrated loop technique was subsequently used for all studies.

Synergy was defined as a 2 log10 or greater decrease in the concentration of bacteria at 20 h with the antimicrobial agent combination compared to the decrease with either antimicrobial agent alone.

**RESULTS**

There was excellent agreement among MIC results obtained by agar dilution and broth dilution (data not shown). Table 1 describes the susceptibility of the 50 strains of enterococci to cefotaxime, gentamicin, tobramycin, and amikacin based on these results. Very few strains of enterococci were inhibited by concentrations of the agents which are achievable in serum. In addition, five strains, all *S. faecalis*, exhibited high-level resistance to aminoglycosides, with MICs greater than 2,048 µg/ml. Only four strains of enterococci were inhibited by less than 62.5 µg of cefotaxime per ml.

Only three strains of enterococci were killed by 250 µg of cefotaxime per ml. The remainder required greater concentrations or were not killed at all. The 50% MBC for gentamicin was 100 µg/ml for tobramycin it was 50 µg/ml, and for amikacin it was greater than 200 µg/ml. The 90% MBC was greater than 200 µg/ml for all three aminoglycosides.

Utilizing time-kill curves, concentrations of antimicrobial agents were chosen on the basis of achievable serum levels or one-fourth the MBC, whichever was lower. The combination of cefotaxime and gentamicin was bactericidal against 16 strains or 35% of the 46 isolates. Cefotaxime plus tobramycin was bactericidal against eight strains or 17%, and cefotaxime plus amikacin was bactericidal against seven strains or 15%. Results with the four strains of *S. faecium* revealed only one strain which was killed by the combination of cefotaxime and gentamicin.

Figure 1 is a typical time-kill curve, utilizing an isolate of *S. faecalis*. In this example, cefotaxime plus gentamicin was the only combination that caused a 2 log10 decrease in colony count at 20 h. Table 2 shows the mean colony counts at 20 h for all enterococci with the different drugs singly and in combination. The combination of cefotaxime and gentamicin resulted in a mean colony count less than other combinations or drugs singly, but the differences were not significant.

**DISCUSSION**

Enterococci account for an important "gap" in the antimicrobial activity of the newer third-generation cephalosporins, and enterococcal superinfection has been described in a number of patients treated with these agents (4, 5, 8; C. D. Schwigon and D. Barckow, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 819, 1982).

Since some combinations of beta-lactam agents and aminoglycosides exhibit synergistic killing against some enterococci, we decided to determine whether the combination of cefotax-
FIG. 1. Typical time-kill curve for enterococci with no antibiotics. Control (●); cefotaxime, 100 μg/ml (○); amikacin, 20 μg/ml, plus cefotaxime, 100 μg/ml (■); amikacin, 20 μg/ml (△); gentamicin, 5 μg/ml (▲); tobramycin, 5 μg/ml (□); tobramycin, 5 μg/ml, plus cefotaxime, 100 μg/ml (△); and gentamicin, 5 μg/ml, plus cefotaxime, 100 μg/ml (○). Only gentamicin plus cefotaxime (○) produced a greater than 2 log₁₀ decrease in colony counts at 20 h and therefore fulfilled the criteria for synergy.

Antimicrobial agents which were chosen for use in the time-kill studies were one-fourth that required to kill the enterococcus (25% MBC) or the usual achievable serum levels, whichever was lower. The concept of in vitro antimicrobial synergism is a complex and ill-defined one. Synergism may be inferred when agents are utilized in a time-kill curve at one-fourth the concentration required for the individual agents to kill the microorganism, and the titer of microorganisms decreases by at least 2 logs in 24 h (2). Other techniques for determination of synergism include the so-called checkerboard method, utilizing broth dilution or time-kill studies based on MICs.

In the in vitro system chosen, synergistic kill of 35% of the strains of S. faecalis occurred with the combination of cefotaxime and gentamicin. Killing was less with cefotaxime combined with tobramycin or amikacin.

Five strains of S. faecalis demonstrated high-level resistance to gentamicin, four of these to amikacin as well. We did not test for high-level resistance to tobramycin. None of these five strains was synergistically killed with time-kill testing by any of the combinations.

It is important to emphasize that some investigators have noted lack of correlation between results of in vitro susceptibility testing of enterococci and in vivo results. For example, a study by Weinstein and Letnick demonstrated the failure of low-dose cephalothin plus gentamicin to prolong survival in rabbits with enterococcal endocarditis even when the infecting strain was susceptible to "synergistic" killing in vitro. When a higher dose of the cephalosporin was used along with aminoglycosides, there was cure in some animals (7).

Since the combination of a third-generation cephalosporin and an aminoglycoside may often be utilized as initial therapy for patients with suspected or proven serious bacterial infectious disease, it is important to determine whether this regimen is active against enterococci. Our in vitro data suggest that the combination of cefotaxime and gentamicin may be effective in some instances. Certainly if enterococci were suspected or proven to be the proximate cause of serious bacterial disease a different therapeutic regimen should be chosen. High-dose intravenous penicillin or ampicillin plus gentamicin is the treatment of choice for enterococcal disease, especially when bactERICidal activity is necessary such as in patients with intravascular infection or in granulocytopenic patients. The new extended-spectrum penicillins such as mezlocillin, azlocillin, and piperacillin have been reported to have more activity against enterococci than the older drugs carbenicillin and ticarcillin. Together with aminoglycosides the new agents may be more active against enterococci than third-generation cephalosporins.

A number of reports have described enterococcal superinfection in patients who received a third-generation cephalosporin alone (4, 5, 8; 22nd ICAAC, abstr. no. 819). It is possible that when a third-generation agent is combined with an aminoglycoside enterococcal superinfection may be less.

To summarize, physicians may anticipate the emergence of enterococci as important opportunistic pathogens in seriously ill patients receiving newer beta-lactam antimicrobial agents. A combination of a third-generation cephalosporin

| Antimicrobial agent          | Conc (μg/ml) | CFU/ml   |
|------------------------------|--------------|----------|
| Cefotaxime                   | 100          | 6.3 × 10⁶|
| Gentamicin                   | 5            | 2.3 × 10⁵|
| Tobramycin                   | 5            | 2.0 × 10⁵|
| Amikacin                     | 20           | 1.3 × 10⁵|
| Cefotaxime + gentamicin      | 100 + 0.3-5  | 4.3 × 10⁶|
| Cefotaxime + tobramycin      | 100 + 5      | 6.6 × 10³|
| Cefotaxime + amikacin        | 100 + 20     | 2.5 × 10⁶|
and an aminoglycoside appears to be marginally effective in vitro against some strains of enterococci. Extensive clinical experience will be required to define the role of this combination for prevention or treatment of enterococcal disease.

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