Synergism of Cefazolin-Gentamicin Against Enterococci

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A miniaturized technique for detecting antibiotic synergy by using microliter volumes was perfected. With this microtiter serial dilution method, the effect of the previously untried combination of cefazolin and gentamicin on 10 strains of enterococci was evaluated. At clinically achievable concentrations, the combination of cefazolin and gentamicin was found to be synergistic against all 10 strains when the data were plotted in the form of an isobologram. These results were compared with the conventional method of detecting synergy in vitro, namely, the determination of the rate of killing of the microorganisms with the antibiotics, singly and in combination. Similar results were also observed. These findings indicate that the microtiter serial dilution technique is a useful method for the routine determination of drug synergy. Moreover, in a patient with penicillin hypersensitivity and enterococcal infection, the combination of cefazolin and gentamicin should be considered for possible therapy.

As microorganisms are encountered that are resistant to certain antibiotics, the use of drug combinations in certain clinical situations has proved valuable. Of particular interest in recent years has been the phenomenon of drug synergism.

Drug synergism, as defined, implies the ability of two antimicrobial drugs acting together to produce a bactericidal activity greater than the sum of the effects produced by each of the drugs independently (6, 11).

Unfortunately, most of the quantitative techniques for detecting drug synergy are laborious and impractical for routine use in clinical laboratories. One purpose of this study was to compare a microtiter method with the conventional method of detecting synergy. The greater efficiency and economy of the microtiter method might implement synergy assays as a routine procedure.

The combination of penicillin and streptomycin has been widely used in the treatment of enterococcal infections since the discovery that these drugs were at times synergistic against such organisms. In recent years, however, this combination of drugs has been found to be ineffective against increasing numbers of enterococci (10, 20, 21), and, hence, an alternative drug regimen would be very valuable.

The combination of penicillin and gentamicin has been shown to be synergistic in its killing action against virtually all strains in vitro (4, 15, 20). Because recent clinical studies have confirmed the in vivo efficacy of gentamicin and penicillin against enterococcal infections, it has been suggested by some investigators that this combination be the initial treatment of choice (17, 19).

However, occasional patients will present such unique circumstances as penicillin allergy. More toxic antibiotics, such as vancomycin, have been successfully used as alternatives in some of these patients (2), but new forms of safe and effective therapy are needed. Cephalosporin derivatives are similar to the penicillins in molecular structure and antibacterial activity, but cross-sensitivity between these compounds and penicillin only rarely occurs (1, 16). For these reasons it was decided to investigate the in vitro activity of a cephalosporin derivative, cefazolin, combined with gentamicin.

In this study, two major goals were attained: a test of the performance of the microtiter serial dilution synergy technique, as well as an evaluation of this previously untried drug combination against enterococci.

MATERIALS AND METHODS

Bacteria. Seven strains of enterococci were obtained from routine cultures of urine, wounds, and blood performed in the clinical microbiology laboratory at John Dempsey Hospital of the University of Connecticut. An additional three strains of enterococci were obtained from the Connecticut Department of Health Laboratory and from the clinical laboratory at Hartford. All 10 enterococci were Streptococcus faecalis. All strains of enterococci were confirmed in the clinical laboratory by routine techniques. The nonenterococcal group D strepto-
coccus (S. bovis) was not included in this study due to its uniform susceptibility to penicillin.

Media and antibiotics. Culture media used were Mueller-Hinton broth (MHB) (BBL) and Mueller-Hinton agar. Antibiotics used in this study were obtained from the hospital pharmacy as injectable solutions without preservative. These antibiotics consisted of gentamicin sulfate (Schering Laboratories) and cefazolin sodium (Eli Lilly & Co.). Stock solutions of the antibiotics were prepared in concentrations of 1,280 μg/ml. These solutions were frozen and stored in 2-ml aliquots at -20 C. The potency of these antibiotics was checked weekly by comparing the minimal inhibitory concentration (MIC) of each drug alone, by using a standard enterococcus, with the MIC of that strain each week thereafter. No variation in the MIC was noted throughout the length of this study.

Microdilution assay for antibiotic synergy. The checkerboard tube dilution technique described by Sabath (11) was adapted to the microtiter system (8) using Autotiter plates containing 120 wells (Ames Co.). After the addition of 50 μl of growth media (MHB) to each of 64 wells (8 x 8 square), antibiotics were serially diluted with a 50-μl spiral loop, horizontally and vertically. Eight rows in one direction contained twofold dilutions of cefazolin in decreasing concentrations ranging from 32 to 0.25 μg/ml, whereas the eight rows in the other direction contained serial twofold dilutions of gentamicin ranging in concentrations from 32 to 0.25 μg/ml. The final result was 64 wells, each containing a different concentration of the cefazolin-gentamicin combination (see Fig. 1). Two additional rows contained twofold serial dilutions of cefazolin and gentamicin alone in final concentrations of from 32 to 0.25 μg/ml. An overnight broth culture of the particular strain was standardized with a nephelometer to 1 x 10⁸ organisms per ml and then diluted to a final inoculum size of 5 x 10⁸ colony-forming units per ml.

One row in each Autotiter plate contained only MHB and bacteria as a growth control, while a second row contained only MHB as a contamination control. Bacteria and antibiotic solutions were added to the wells of the Autotiter tray with a calibrated 50-μl pipette. The final volume in each well was 100 μl.

Each Autotiter tray was sealed with plastic transparent tape and incubated at 37 C for 18 to 24 h. After incubation, the trays were examined for evidence of growth by using the Autotray Viewer (As-tec, Inc.). The turbidity of the wells containing only enterococci and broth was used for comparison in determining the end points of growth. The lowest concentration of antibiotics showing complete inhibition of growth was read as the MIC (micrograms per milliliter).

For determination of bactericidal end points, samples were removed from each row with a 0.01-ml platinum-calibrated loop, from the end point well and from the other five wells on either side. These samples were plated out on Mueller-Hinton agar and observed for evidence of growth after incubation at 37 C for 18 to 24 h. The lowest dilution in which there was no growth was noted as the minimum bactericidal concentration (MBC) in micrograms per milliliter.

The MIC and MBC of each antibiotic, alone and in combination, were recorded and plotted as an isobologram on an arithmetic scale. Concave curves represent synergistic action of the two drugs, a straight line joining the ordinate and abscissa axes represents an additive effect, and a convex outward curve represents antagonism of the two drugs (11).

Growth curve and assay for synergy. The rate of killing of the enterococci by cefazolin and gentamicin, alone and in combination, was determined for all 10 strains by performing serial colony counts. Overnight broth cultures for each strain were standardized with a nephelometer to 1 x 10⁸ organisms per ml. The final concentration range of each antibiotic was then added to the tubes, and the tests were performed as described above.

Fig. 1. Diagram of an Autotiter tray with the different concentrations of antibiotics in each well. C, Cefazolin; G, gentamicin in micrograms per milliliter.
per ml and diluted, and 2-ml aliquots of these organisms were added to a series of tubes to reach a final inoculum size of approximately $5 \times 10^6$ organisms per ml. Antibiotics were added, singly and in combination, to these tubes so that the final concentration would be as follows: cefazolin, either 32 $\mu$g/ml (five strains) or 16 $\mu$g/ml (five strains); gentamicin, either 4, 2, or 1 $\mu$g/ml. The concentrations of gentamicin were chosen to be less than the MIC (previously determined for each particular strain by the microtiter serial dilution technique) for, in order to demonstrate a synergistic effect, unimpaired growth in the presence of gentamicin alone was required. Each strain was therefore tested against the two antimicrobials singly and against two to three distinct concentrations of gentamicin in combination with a single concentration of cefazolin. An additional test tube contained only MHB and bacteria and served as a growth control. The final total volume in each tube was 4 ml.

The tubes were incubated at 37 C for 4, 8, and 24 h. One-milliliter samples were removed at each time period, 10-fold serial dilutions were made, and colony counts were performed on Mueller-Hinton agar. The plates were then incubated at 37 C and the colonies were counted after 24 h of incubation. According to the criterion used by Watanakunakorn (15), the combination of antibiotics was considered synergistic when the number of colonies surviving the combined killing action was at least 1 log$_{10}$ less than the number of colonies surviving with the most effective of the antibiotics alone.

RESULTS

The MIC and MBC of cefazolin and gentamicin, alone and in combination, against 10 strains of enterococci are illustrated in Fig. 2 and 3. The lines joining the MICs and MBCs of the various combinations (isobols) for all 10 strains are distinctly concave and, according to the criteria of Sabath (11), represent synergy of the two antibiotics. Due to the overlap of many of the isobols in Fig. 2 and 3, Fig. 4 has been included to present a clearer visualization of a single isobol from a single strain (15). The concave nature of the curve signifies synergy. These results are summarized in Table 1.

The activity of cefazolin and gentamicin alone and in combination against the 10 strains

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**Fig. 2. Isobolograms of MICs for enterococcal strains 1-10.**
of enterococci was evaluated in broth culture. The results are presented in Table 2. Using the criteria of synergy as a decrease in viable cell numbers after 24 h of incubation by at least a factor of 10, when the combination of drugs is compared to the most effective of the antibiotics alone, all 10 strains (100%) demonstrated synergy. An example of a growth curve showing synergy (enterococcus no. 4) is presented in Fig. 5. The concentrations of gentamicin (4 and 2 \( \mu g/ml \)) were chosen because the MIC of gentamicin for this particular strain was found to be \( 8 \mu g/ml \) by a previous determination, and, in order to demonstrate the synergistic effect, unimpaired growth in the presence of gentamicin alone was required.

**DISCUSSION**

Two methods for evaluating synergy between cefazolin and gentamicin were evaluated in this study. The microtiter serial dilution method yields results that indicate whether there is synergy, antagonism, or an additive effect of antibiotic combinations. This technique also provides quantitative data in the form of the MIC and MBC of each antibiotic for the microorganisms being considered. This method proved to be a rapid, efficient, and economical method of assay for synergy. With the availability of equipment that will automate this miniaturized checkerboard technique, routine determinations of antibiotic synergy may soon be feasible in many clinical laboratories.

The conventional large-volume, broth dilution technique with the plotting of growth curves used in the second part of this study is a cumbersome, time-consuming, and expensive technique. The results of this study show a good agreement between these two techniques. Because many other studies have also attested to the accuracy and reproducibility of the microdilution technique when compared to the broth dilution method (3, 7, 13), serious consideration should be given to its implementation.

The exact mechanism of the penicillin-streptomycin synergy against the enterococci is not known. However, it has been shown that penicillin increases the uptake of radioactive streptomycin by enterococci, presumably as a result of injury to the cell wall (9). However, despite
penetration into the cell by streptomycin, increasing numbers of enterococcal strains remain unaffected by even high levels of this antibiotic when combined with penicillin (3, 10). This high level of resistance has been found to be ribosomally mediated (21).

To date, because ribosomally mediated resistance has not been observed with gentamicin and because virtually all strains of enterococci are susceptible to the synergistic bactericidal effects of the combination of gentamicin and penicillin (15, 20), this combination emerges as an attractive choice in the therapy of enterococcal endocarditis (17). However, it must be stressed that in vitro evidence of synergism is not always predictive of therapeutic success and, hence, 20 to 40 million U of penicillin G intravenously plus 1 g of streptomycin intramuscularly probably remains the preferred selection in the initial treatment of enterococcal endocarditis; however, if the clinical response is poor, then prompt consideration

TABLE 1. Summary of isobolograms for detecting synergism between cefazolin and gentamicin against 10 enterococcal strains

<table>
<thead>
<tr>
<th>Antibiotic activity</th>
<th>No. of enterococcal strains</th>
<th>Shape of isobologram</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concave</td>
<td>Linear</td>
</tr>
<tr>
<td>MIC</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>MBC</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE 2. Effectiveness of combination of cefazolin and gentamicin against 10 strains of enterococci as determined by growth curves

<table>
<thead>
<tr>
<th>No. of enterococcal strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1&quot;</td>
</tr>
<tr>
<td>1-2</td>
</tr>
<tr>
<td>2-3</td>
</tr>
<tr>
<td>3-4</td>
</tr>
<tr>
<td>4-5</td>
</tr>
</tbody>
</table>

" Numbers show fold log₀ decrease in the number of surviving colonies. Decrease in viable cell count after 24 h by the combination was compared with the most effective of the antibiotics used alone. In all cases, the most effective antibiotic alone was cefazolin since the aminoglycoside concentration was chosen to be less than the MIC.
should be given to the replacement of the streptomycin with gentamicin in appropriate dosage (19).

In patients allergic to the penicillins, treatment of systemic enterococcal infections becomes more complex. In the past, various therapeutic approaches have been tried, such as substituting a more toxic agent for penicillin, attempting to desensitize the patient, or using corticosteroids and antihistamines to depress the allergic response (5).

Since cephalosporin derivatives have become available, they represent a potentially useful alternative to penicillin treatment of enterococcal infections. These antibiotics are relatively nontoxic and have an antibacterial spectrum similar to penicillin G, and cross-hypersensitivity reactions between the penicillins and the cephalosporins only rarely occur (1, 16). Previous studies have shown the combination of cephalothin, another cephalosporin derivative, and streptomycin to be a relatively ineffective combination against enterococci (12). However, the results of more recent studies clearly demonstrate the superiority of gentamicin against enterococci when coupled with other antibiotics (15, 20). The results of this present study using two different techniques show a marked synergistic effect when gentamicin was combined with the cephalosporin derivative cefazolin. At clinically achievable concentrations of these antibiotics, synergy was demonstrated with all 10 strains, with a 100% correlation of the results between the two techniques. Our results were not surprising since it has been shown in a previous study that the combination of cephalothin and gentamicin was also synergistic against strains of enterococci (18).
These in vitro results suggest that cefazolin offers promise as a penicillin substitute for the treatment of enterococcal endocarditis.

LITERATURE CITED