Synergism of Carbenicillin and Gentamicin Against Enterococci

ROBERT D. LIBKE, CLAUDE REGAMEY, JOHN T. CLARKE, AND WILLIAM M. M. KIRBY

Department of Medicine, University of Washington School of Medicine, Seattle, Washington 98195

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Carbenicillin and gentamicin were tested for synergism against 25 strains of enterococci by two different methods. Killing curves were determined by doing serial colony counts of broth cultures containing the antibiotics separately and in combination. The combination was synergistic for all 25 strains with 75 and 4 μg of carbenicillin and gentamicin per ml, respectively. Reducing the concentrations to 50 and 3 μg of the antibiotics per ml with four strains significantly reduced the rate of killing of the combination. Synergism was also studied by constructing isobolograms, by using the standard two-dimensional broth dilution checkerboard technique, and by measuring the end points both for minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs). Synergism was present for all 25 strains when bactericidal end points (MBCs) were evaluated, but was present for only 7 of the 25 strains when MICs were used to construct isobolograms. The time and effort involved were roughly the same for killing curves and for isobolograms, and it was concluded that neither had a distinct advantage over the other.

Enterococci are often implicated as causative agents in infections involving the endocardium, abdomen, pelvis, and urinary tract. Combinations of antibiotics, especially penicillins and aminoglycosides, have been found to provide the bactericidal action often required to achieve a clinical cure (16). The mechanism responsible for this synergistic action involves alteration of permeability of the bacterial cell wall by the penicillin so that the aminoglycoside can penetrate the organism and exert its effect on the ribosomes (18).

Carbenicillin and gentamicin are frequently administered to patients with presumed sepsis and have been shown to have a synergistic action against Pseudomonas and other gram-negative bacteria (5, 14). The present study was undertaken to determine in vitro whether carbenicillin and gentamicin in concentrations usually obtained in the blood of patients would show a synergistic, bactericidal action against enterococci. In addition to the usual method of measuring the rate of killing by performing colony counts, synergism was also studied by constructing isobolograms and measuring both bacteriostatic and bactericidal end points. Streptomycin minimal inhibitory concentrations (MICs) were also measured to determine the number of strains susceptible to gentamicin but not to streptomycin.

MATERIALS AND METHODS

Antibiotics. Carbenicillin and gentamicin standard powders were supplied by Pfizer, Inc. and Schering Corp., respectively.

Bacteria. Twenty-five strains of enterococci, isolated from a variety of clinical sources, were obtained from the clinical microbiology laboratory at the University of Washington Hospital. They were identified by colonial appearance on blood and MacConkey agar and by their ability to ferment mannitol and reduce 0.1% methylene blue milk. The strains were grown in Mueller Hinton Broth (MHB) prior to use and streaked onto blood and MacConkey agar to check for contamination before testing.

Media. Mueller Hinton broth (Difco) adjusted to pH 7.4 was used for cultures and serial dilutions of the antibiotics and bacteria.

Synergism as determined by killing curves. Serial colony counts were performed with broth cultures of the 25 strains of enterococci to determine the time-related bactericidal effects of carbenicillin and gentamicin alone and in combination (3, 7). A 1-ml portion of a 10⁻³ dilution of an overnight culture was added to 9 ml of broth containing one or both antibiotics to give an average final inoculum of 4.6 × 10⁶ organisms per ml (range 2.53 to 5.80 × 10⁶ for the 25 strains). The final antibiotic concentrations were 75 and 4 μg/ml for carbenicillin and gentamicin, respectively, and four strains were also tested with 50 and 3 μg/ml. At 0, 2, 4, 8, and 24 h, 0.1 ml from each tube was transferred undiluted or in 10-fold dilutions in normal saline to petri dishes, and pour plates were
made with Trypticase soy yeast (TSY) agar. Because only 0.1 ml was sampled, 10 colony-forming units (CFUs) per ml was the lowest number detectable so that a zero colony count represented a range of no survivors to as many as nine viable units per ml. Synergism with this method was defined by the commonly accepted criterion as a decrease of 1 log or more in the colony count at the end of 4 h with the combination as compared with the count obtained with the most active single agent (carbenicillin) (1, 3). Because the rate of growth of the bacteria in the absence of antibiotics was not involved in the criterion for defining synergism, and because of the relatively uniform growth of all the strains in broth without antibiotics, as evidenced by the narrow range in counts of undiluted cultures (2.53 to 5.8 x 10^9) after overnight growth, serial colony counts of control tubes were performed on only one of the four strains tested each day.

Synergism as determined by isobolograms. Isobolograms were constructed for the 25 strains from the MICs and minimal bactericidal concentration (MBCs) determined by the standard two-dimensional broth dilution checkerboard method (6, 10, 12, 14). Final concentrations of 0.125 to 128 μg/ml of gentamicin per ml and 0.5 to 512 μg/ml of carbenicillin per ml, alone and in all possible combinations, were obtained by adding 0.5 ml of 10^-2 dilutions of overnight broth cultures to 0.5 ml of broth in tubes containing serial twofold dilutions of carbenicillin in rows of 12 on one axis and of gentamicin on the other (144 tubes in all). The MIC was defined as the lowest concentration that prevented gross turbidity upon visual inspection after incubation at 37°C for 18 to 20 h. From the clear tubes 0.01 ml of broth was removed via a calibrated loop and streaked on a TSY agar plate, and the MBC was designated as the lowest concentration in which fewer than 0.1% of the original inocula of organisms remained viable; i.e., 20 CFUs or less in the 0.01-ml portion removed (17). Isobolograms for each strain were constructed by plotting the inhibitory and bactericidal end points on an arithmetic scale in the usual manner, with the carbenicillin and gentamicin concentrations represented on opposing axes. The isobole defining the additive effect of the two antibiotics was obtained by drawing a straight line between the MICs or MBCs of each antibiotic acting alone. Synergism was then evident if the line representing the combined effect of the antibiotics at various concentrations fell below the line for the additive isobole (6, 12).

Variations of one tube in MICs and MBCs are commonly observed with serial dilution tests, and one-tube differences have a marked effect on the shape of the isobologram. As a result, some investigators have defined synergism as at least a two-tube (fourfold) reduction of the MIC or MBC of both antibiotics (4, 10, 17). This causes a greater deviation, from the additive isobole, of the line obtained by connecting the observed end points than would be present if any concave bowing (for example, a twofold reduction with one antibiotic and fourfold with the other) was accepted as evidence of synergy. For the purposes of this study the former, more stringent criteria were used, and synergism was considered present only when there was, with the combination, at least a fourfold reduction in the MIC or MBC of both antibiotics. A twofold reduction of the MIC or MBC of both or either was considered additive, and no lowering of the end points was regarded as indifference (1, 12).

Streptomycin MICS. Previous studies have shown that the rate of killing of strains of enterococci that are highly resistant to streptomycin (MIC > 1,000 μg/ml) is not enhanced by combining this antibiotic with penicillin G (8, 13). On the other hand, strains highly resistant to gentamicin have not been described, and synergism of this antibiotic with penicillin G has invariably been present (9, 15). It seemed of interest, therefore, to determine how many of our 25 strains were resistant to streptomycin and to see if there was any cross-resistance with gentamicin. Mueller Hinton agar (Difco), adjusted to pH 7.4, was used to make plates containing twofold serial dilutions of streptomycin in concentrations from 1.024 to 4 μg/ml and to make a plate without antibiotic as a control. The Steers replicator device was then used to transfer 0.001 to 0.002 ml of a 10^-3 dilution of an overnight culture of each strain onto the surface of the agar plates. The lowest concentration of streptomycin permitting growth of no more than one colony was called the MIC, an end point that has become commonly accepted for the agar dilution technique because of the occurrence of occasional resistant mutants (2, 11).

RESULTS

MICs and MBCs. As seen in Table 1, MICs of carbenicillin for the 25 strains of enterococci ranged from 32 to 256 μg/ml, and 96% were inhibited by 128 μg/ml. The MBCs of carbenicillin varied from 64 to greater than 512 μg/ml. Gentamicin MICs ranged from 4 to 32 μg/ml, and 96% of the strains were inhibited by 16 μg/ml. The MBCs of gentamicin varied from 4 to 64 μg/ml. Only 60% of the strains were inhibited by 1,000 μg of streptomycin per ml, a figure in close agreement with that of other investigators (8). When MICs for streptomycin and gentamicin were plotted against each other there was no correlation between susceptibility or resistance to streptomycin and the degree of susceptibility of individual strains to gentamicin (r = 0.10).

Killing curves. Synergism, as defined in Materials and Methods, was demonstrated for all 25 strains. A typical killing curve for a strain showing a difference of more than 2 logs is presented in Fig. 1. As can be seen in Table 2, all but two of the strains exhibited a decrease of 2 logs or more with the combination at 4 h. A difference of slightly less than 2 logs occurred with the two other strains. Two strains failed to maintain a 1 log difference throughout the entire 24-h test period but, as suggested by Jawetz, this is probably not significant as long
as the increased rate of early killing is present (3). With the antibiotic combination, reduction to the lowest colony count detectable was reached within 8 h for 23 of the 25 strains and within 24 h for all strains. Carbenicillin alone decreased the colony count initially with all but one strain, but not to less than 100 CFUs in any instance during the first 8 h. Growth of the enterococci in most instances was only slightly slowed by gentamicin at 4 \( \mu \text{g/ml} \) (see Fig. 1), and there was a reduction from the initial colony count with only one strain.

Four strains were also tested with lower antibiotic concentrations, namely, 3 and 50 \( \mu \text{g} \) of gentamicin and carbenicillin per ml, respectively. Under these circumstances there was synergism with three of four strains, although the decrease in colony count as compared with carbenicillin alone was only between 1 and 2 logs with two of the three strains (Fig. 2). Furthermore, colony counts with the three strains showing synergism did not decrease to below 100 CFUs, a marked contrast to the results obtained with the higher antibiotic concentrations described above where all 25 strains showed complete killing (reduction to the lowest counts measurable).

**Synergism as determined by isobolograms.** By using the criteria described above, isobolograms obtained by plotting MBCs of the antibiotics combined in different ratios showed synergism against all 25 strains, agreeing completely with the results of the killing curves. Indeed, for 24 of the 25 strains the reduction was much greater than the minimal requirement of a fourfold lowering of the MBCs for both antibiotics. In contrast, synergism was present for only 7 of the 25 strains when bacteriostatic rather than bactericidal end points were used to construct the isobolograms. With 14 strains the effect was additive, and indifference was noted with four strains.

**DISCUSSION**

It has become well established that enterococcal infections, especially endocarditis, are most likely to respond to synergistic combinations of antibiotics. Synergism has been shown to occur against 60% of strains of enterococci when they are exposed to penicillin \( G \) in combination with streptomycin, 80% with penicillin and kanamycin, and 100% with penicillin and gentamicin (8, 9, 15). Carbenicillin and gentamicin were shown in the present study to have synergistic, bactericidal activity against all of 25 strains of enterococci. McCracken et al.
Table 2. Effectiveness of carbenicillin and gentamicin in combination against 25 strains of enterococci

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>No. of logs greater killing by the antibiotic combination compared with carbenicillin alone</th>
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<tr>
<td></td>
<td>0-1</td>
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<tr>
<td>4</td>
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<tr>
<td>8</td>
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<td>24</td>
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* Values represent number of strains.

Fig. 2. Decreased synergistic activity with lowered concentrations of carbenicillin and gentamicin by using the same strain of enterococcus as in the study depicted in Fig. 1.

have recently reported synergism against 10 strains (6).

Gentamicin at 4 μg/ml and carbenicillin at 75 μg/ml were selected as representing concentrations usually attained in the blood of patients being treated for serious infections. With gentamicin alone, MICs were usually 8 or 16 μg/ml (Table 1) and killing curves showed little bactericidal activity at 4 μg/ml (Fig. 1). Carbenicillin MICs were usually 64 or 128 μg/ml, and although there was an initial decrease in colony counts (Fig. 1), complete killing often did not occur even at much higher levels (Table 1). In contrast, the combination caused a decrease in colony counts to zero with all strains. Killing was much less complete when, with four strains, concentrations were decreased to 3 and 50 μg of gentamicin and carbenicillin per ml, respectively, suggesting that full therapeutic doses may be needed if the phenomenon of synergy is likely to be effective clinically.

The demonstration of synergy by killing curves is time consuming, and alternative methods might be preferable. Therefore, attempts were made to correlate the results of killing curves with the other widely used method of measuring synergy, namely, the construction of isobolograms by using broth dilution tests in which the two antibiotics are combined in 144 tubes in a large variety of concentrations, the so-called checkerboard technique (6, 12, 17). Both MICs and MBCs were used to construct isobolograms, and with the latter synergism was present with all 25 strains, a perfect correlation with the results of killing curves. On the other hand, there was synergism with only 7 of the 25 strains when MICs were used as the end points. McCracken et al. did observe synergism against 10 strains of enterococci when MICs were used as the basis for isobolograms. Although their criteria for synergism were less stringent than ours, this does not fully explain the difference, because synergism would be present with only 68% of our strains if the criteria of McCracken et al. were used. Because isobolograms using MBCs correlated with killing curves as noted above, the technique could serve as an alternative method for detecting synergy, although the time and effort were not appreciably less.

The mechanism responsible for synergism against enterococci is probably the same with carbenicillin as for penicillin G and ampicillin, namely, it increases the permeability to the aminoglycoside and thereby facilitates its action on the ribosomes (18). None of our strains was highly resistant to gentamicin, and none has been described so far (9). On the other hand, high-level resistance to streptomycin, present with 40% of our 25 strains, is associated with a lack of synergistic activity, presumably because the organisms are resistant at the ribosomal level even when significant intracellular concentrations are reached (18). This may be because the large, single-step mutations commonly encountered with streptomycin occur rarely, if ever, with gentamicin.

The cost of carbenicillin and the difficulty in maintaining adequate blood levels for long periods of time would make it impractical to treat infections known to be caused only by enterococci with carbenicillin and gentamicin. It is a common practice, however, to use this antibiotic combination in the treatment of patients with possible sepsis pending etiologic diagnosis, especially when an abdominal source is likely. The in vitro synergy demonstrated in
this study indicates that under these circumstances effective antibacterial activity against enterococci should be present.

LITERATURE CITED