Bactericidal Synergy Between Penicillin or Ampicillin and Aminoglycosides Against Antibiotic-Tolerant Lactobacilli

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The bactericidal activities of penicillin G and ampicillin alone were compared with those of their combinations with streptomycin or gentamicin against 17 strains of lactobacilli classified as tolerant to various β-lactam antibiotics. The penicillin G combinations with streptomycin and gentamicin were synergistic against 17 and 16 of these strains, respectively, whereas the corresponding ampicillin-aminoglycoside combinations were synergistic against 12 and 15 strains, respectively. Importantly, synergy was manifested at concentrations of these antibiotics that are attained in serum after their administration in conventional dose regimens. In no instances were combinations antagonistic. These in vitro observations provide a partial explanation for the favorable results obtained in preliminary clinical evaluations of the benefits of combination regimens in the treatment of lactobacillus infections refractory to single-drug therapy.

Serious lactobacillus infections, particularly endocarditis, often result in therapeutic failure when treated with seemingly appropriate single-drug antibiotic regimens, despite readily achievable minimal inhibitory concentrations (MICs) (1, 3, 20). Our previous studies indicated that this disparity between in vitro susceptibility data and in vivo efficacy may be partially explicable on the basis of “antibiotic tolerance” exhibited by a number of Lactobacillus strains (3-5). The marked discrepancy between MICs and minimal bactericidal concentrations (MBCs) for individual Lactobacillus isolates (1, 3-5) and preliminary clinical evaluations suggesting efficacy of penicillin-aminoglycoside regimens in cases of refractory lactobacillus endocarditis (1, 3, 20) prompted the present study to examine the synergistic bactericidal potential of penicillin-aminoglycoside combinations against 17 antibiotic-tolerant Lactobacillus strains.

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MATERIALS AND METHODS

Test organisms. The 17 lactobacilli were clinical isolates recovered from various sources including blood (14 strains), abscess fluid (2 strains), and female genital tract (1 strain). Twelve were obtained from the clinical laboratory of Harbor-UCLA Medical Center, and the remaining five organisms were kindly provided by M. Elisabeth Sharpe, Reading, England.

These lactobacilli were identified by typical appearance on Gram stain and by biochemical reactions according to Bergey’s criteria (17). Identification of species was according to classifications of Holdeman and Moore (12). The following strains were used: L. casei (12 strains), L. plantarum (3 strains), and L. brevis (2 strains). The L. casei and L. plantarum isolates were facultative anaerobes, and the L. brevis strains were strict anaerobes.

Organisms were maintained in chopped meat-glucose broth (Scott Laboratories, Fiskeville, R.I.) and were transferred monthly into fresh media until the susceptibility testing was performed. In the week before testing, one additional transfer was done. All susceptibility studies were performed anaerobically.

Antibiotic tolerance. These 17 isolates were confirmed as penicillin and ampicillin tolerant by the following criteria: (i) MBC/MIC ratios of ≥32:1 (18); and (ii) slow bactericidal effect on timed-killing analysis (<99.9% killing at 24 to 48 h).

MBC/MIC ratios. MICs and MBCs were determined for penicillin G and ampicillin by a modified broth dilution technique as previously described (4), using prereduced Mueller-Hinton (MH) broth (BBL Microbiology Systems, Cockeysville, Md.) with 0.2% yeast extract and 0.05% l-cysteine. This medium has a maximal concentration of 0.5 mg per 100 ml of magnesium and 3.1 mg per 100 ml of calcium.

Penicillin G and ampicillin were supplied by USP Standards, Rockville, Md. Stock solutions (8,000 µg/ml) of each agent were prepared and stored at −20°C until the day of the study. Serial twofold dilutions were made of both antibiotics and added to broth under anaerobic conditions. A final inoculum of approximately 2 × 10³ colony-forming units (CFU) per antibiotic-containing tube was used (3-5). After addition of the lactobacillus inoculum, the final range of drug concentrations was 0.312 to 320 µg/ml for each agent. The MIC was read at 24 h as the lowest concentration of penicillin G and ampicillin required to inhibit visible growth. For determination of MBC, 0.01 ml was removed from each clear tube at 24 h, streaked onto antibiotic-free blood agar plates, and incubated anaerobically for 48 h at 37°C. The MBC was defined
as the lowest antibiotic concentration necessary to achieve >99.9% killing.

An L. plantarum isolate with known penicillin G and ampicillin MICs and MBCs was included in each determination for reproducibility.

**Timed-kill assays (tolerance curves).** Timed-kill assays or tolerance curves were constructed after the method of Bradley et al. (8). Briefly, serial dilutions of the penicillin G and ampicillin stock solutions were made in MH broth to a concentration of 20 μg/ml. An overnight MH broth culture of each isolate to be tested was adjusted to a McFarland no. 1 nephelometer standard (2) previously determined to approximate 4 × 10^8 CFU/ml. Nephelometer approximations were routinely confirmed by formal dilution plate colony counts.

Inocula of approximately 4 × 10^6 CFU were added to the antibiotic-containing tubes to achieve a final penicillin G or ampicillin concentration of 10 μg/ml and a final inoculum of approximately 2 × 10^8 CFU. The same inoculum was added to the MH broth containing no antibiotics as a growth control. A Lactobacillus strain known to be rapidly killed by penicillin G and ampicillin at levels of ≤10 μg/ml was also included in this timed-kill assay system as a nontolerant control.

Just before incubation, a 0.01-ml portion was subcultured from each antibiotic-containing and control tube onto antibiotic-free blood agar plates as a 0-h base line; all tubes were then incubated at 37°C. At 6, 24, and 48 h postincubation, all tubes were similarly subcultured onto solid media. All subculture plates were incubated at 37°C for 48 h. Positive penicillin G-ampicillin tolerance was defined as <99.9% of the 0-h base-line colony counts at 24 to 48 h (8). Since antibiotic tolerance has occasionally been noted to wane after storage of the organisms or their passages in media, all isolates were confirmed as tolerant just before synergy testing (C. G. Mayhall, personal communication).

**Synergy testing.** Bactericidal synergy testing of combinations of penicillin G or ampicillin plus streptomycin or gentamicin against tolerant lactobacilli was performed by the microtiter checkerboard technique (16). For penicillin G and ampicillin, the final drug concentration range in the microtiter wells was 0.03 to 80 μg/ml; for streptomycin, this range was 5 to 320 μg/ml; and for gentamicin, the range was 0.312 to 20 μg/ml. These ranges were selected to encompass concentrations for each drug which are readily obtainable in serum after administration in conventional dose regimens (penicillin G-ampicillin, ≤10 μg/ml; streptomycin, ≤20 μg/ml; gentamicin, ≤3 μg/ml [7, 9, 11]).

For each Lactobacillus isolate, 0.1-ml portions of the chopped meat-glucose broth maintenance cultures were added to prerduced MH broth. This was incubated for 24 h at 37°C and adjusted to a McFarland no. 1 nephelometer standard (2) previously determined to approximate 4 × 10^8 CFU/ml. The 24-h MH broth cultures were diluted 1:100 to an inoculum of approximately 4 × 10^6 CFU/ml; each well with 50 μl of antibiotic-containing MH broth was then inoculated with 50 μl of the bacterial suspension to achieve a final inoculum of approximately 10^8 CFU. One well of the microtiter plate contained only broth, without antibiotic, as a positive control for lactobacillus growth. All plates were sealed with sterile plastic lids and incubated at 37°C for 24 h in anaerobic incubation jars. The MBC of each drug alone and the bactericidal effect of the penicillin-aminoglycoside combinations were determined by subculturing 25 μl of the contents of each well onto sterile, antibiotic-free blood agar plates, using a micropipette. These plates were incubated at 37°C for 48 h. The MBC was defined as the lowest antibiotic concentration yielding two or fewer colonies on subculture (>99.9% killing). Synergistic bactericidal activity was considered present (positive) if the MBCs for both drugs tested in combination were reduced fourfold or greater from the MBCs of the individual drugs. “Partial synergy” was defined as a twofold reduction in the MBC of one drug in a combination, with a fourfold or greater reduction in MBC of the other drug. “Indifference” was defined as no more than a twofold reduction in the MBCs of both drugs in combination or at least a fourfold reduction of one drug in a combination with no change in the MBC of the second drug. “Antagonism” was defined as an increase in either or both of the single-drug MBCs in a particular combination. “Synergy range” is defined as the drug concentration range over which synergistic bactericidal activity was observed for an individual antibiotic in a two-drug combination.

**Statistical analyses.** The chi-square test with Yates correction was used in comparative assessments of antibiotic combinations.

**RESULTS**

Penicillin-ampicillin broth dilution MICs and MBCs. The penicillin G and ampicillin MICs for these 17 tolerant lactobacilli were within readily achievable serum levels for both drugs; the MIC range was 0.035 to 2.5 μg/ml for penicillin G and 0.15 to 2.5 μg/ml for ampicillin. In contrast, all MBCs for penicillin G and ampicillin were above serum levels which are generally obtained with conventional dose regimens of these two drugs; the range of MBCs was 10 to 80 μg/ml for each agent. MBC/MIC ratios were ≥32:1 for both penicillins, ranging from 32:1 to ≥133:1 for penicillin G and 32:1 to ≥267:1 for ampicillin.

**Timed-kill assays (tolerance curves).** Figure 1 demonstrates a 48-h tolerance curve for Lactobacillus isolate 1, incubated in 10 μg of both penicillin G and ampicillin per ml. As is typical of tolerant bacterial strains, there was a slow bactericidal effect seen over 24 to 48 h, with <99.9% killing of the base-line inoculum. Similar curves were observed for the remaining 16 Lactobacillus isolates, confirming tolerance to penicillin G and ampicillin. In contrast, base-line colony counts of the control, nontolerant Lactobacillus strain were rapidly reduced by >1,000-fold at 24 h postincubation in antibiotic-containing tubes.

**Synergy testing.** (i) Single-drug MBCs. Single-drug MBCs for penicillin G and ampicillin-
lin performed by microtiter technique during synergy testing were comparable to those determined by broth dilution. All 17 isolates were resistant to the bactericidal effect of streptomycin (MBC, ≥20 μg/ml). For gentamicin, only 3 of 17 isolates (18%) were moderately susceptible (MBC, 1.25 to 2.5 μg/ml). The remaining strains were resistant to killing by gentamicin (MBC, ≥5 μg/ml).

(ii) Drug combination MBCs. Penicillin G in combination with either streptomycin or gentamicin demonstrated markedly enhanced bactericidal activity. The combination of penicillin G plus streptomycin was synergistically bactericidal against all 17 Lactobacillus strains. Significantly, for both penicillin G and streptomycin, synergy was noted over a concentration range which is generally attainable in serum, using standard dosage regimens; for penicillin G the synergy range was 0.035 to 5 μg/ml, whereas for streptomycin this range was 5 to 20 μg/ml. Penicillin G-plus-gentamicin combinations were synergistically bactericidal against 16 of 17 strains at drug concentrations for each agent that are readily achievable in serum; for penicillin G the synergy range was 0.07 to 10 μg/ml, and for gentamicin it was 0.31 to 5 μg/ml.

Ampicillin in combination with either streptomycin or gentamicin also demonstrated excellent bactericidal activity, although somewhat less so than penicillin G-aminoglycoside combinations. Ampicillin-plus-streptomycin combinations were synergistically bactericidal against 12 of 17 isolates (70%), whereas ampicillin-plus-gentamicin combinations were synergistically bactericidal against 15 of 17 isolates (88%). For both ampicillin-aminoglycoside combinations, synergistic bactericidal activity was seen at drug concentrations which are readily achievable in serum with standard parenteral dosages of each antibiotic agent. With ampicillin-streptomycin combinations, the synergy range was 0.035 to 10 μg/ml for ampicillin and 5 to 20 μg/ml for streptomycin; with ampicillin-gentamicin combinations, the synergy range was 0.07 to 10 μg/ml for ampicillin and 0.31 to 5 μg/ml for gentamicin.

Antagonism was not observed in tolerant Lactobacillus strains not synergistically killed by the various β-lactam-aminoglycoside combinations. These strains showed either partial synergy or indifference (Fig. 2).

Statistical comparisons. Combinations of penicillin G plus either streptomycin or gentamicin demonstrated equal bactericidal activity. However, penicillin G-streptomycin combinations were statistically better than ampicillin-streptomycin regimes in this regard (P < 0.05).

DISCUSSION

Clinically, lactobacillemia associated with deep-seated foci, particularly endocarditis, has been relatively refractory to high-dose parenteral therapy with the penicillins, despite MICs which are readily attainable in serum (1, 13, 19, 20). Our previous studies have demonstrated that this discrepancy between in vitro activity and suboptimal therapeutic responses in vivo may be related to antibiotic tolerance of lactobacilli to β-lactam and other antibiotics. MBC/MIC ratios for the lactobacilli in our previous studies have ranged from 20:1 to 30:1 for cephalothin, cefazolin, cefamandole, and cephaloridine, 62:1 for clindamycin, and 32:1 to 267:1 for penicillin G and ampicillin (3-5). Moreover, for the β-lactam antibiotics, MBCs have exceeded their corresponding MICs by ≥100-fold in 47 to 64% of paired MBC-MIC determinations (3, 4). Timed-kill tolerance curves in the present study have confirmed that these lactobacilli with widely disparate MBC/MIC values do in fact exhibit a slow bactericidal effect in the presence of β-lactam antibiotics, typical of tolerant microorganisms.

A similar in vitro phenomenon has now been shown for a variety of other gram-positive pathogens such as the group B and D streptococci, Staphylococcus aureus, and Listeria monocytogenes (8, 13-15, 18). Penicillin-aminoglycoside combinations have demonstrated synergistic in vitro or in vivo efficacy, or both, against these pathogens (4, 13-15). In addition, preliminary synergistic in vitro data from our laboratory, as
Fig. 2. Percentage of reduction of single-drug MBCs by combinations of (A) penicillin G plus gentamicin, (B) penicillin plus streptomycin, (C) ampicillin plus gentamicin, and (D) ampicillin plus streptomycin. Symbols: □, Antagonism; ○, partial synergy; ●, indifference; ◆, synergy.

well as limited clinical experiences, have also suggested that such combinations might be efficacious against the lactobacilli (1, 3, 20).

The present study indicates that penicillin G or ampicillin-aminoglycoside combinations also demonstrate excellent bactericidal activity in vitro against β-lactam-tolerant lactobacilli. Among the 17 lactobacilli tested, synergistic bactericidal activity was demonstrated in 94 to 100% of assays using penicillin G-aminoglycoside combinations. Of importance, the concentration range over which bactericidal activity occurred consistently encompassed readily achievable serum levels for both drugs in the combinations. The mechanism of the apparent superiority of penicillin G versus ampicillin in synergy testing against antibiotic-tolerant lactobacilli is not known.

A variety of single-antibiotic regimens have been efficacious in the treatment of most lactobacillus infections (3). However, the frequent failure of these agents to cure lactobacillus endocarditis and the synergy data presented in this study indicate that penicillin-aminoglycoside
combination chemotherapy is the regimen of choice for deep-seated or refractory lactobacillus infections, particularly endocarditis.

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LITERATURE CITED