Effective Treatment of Multidrug-Resistant Enterococcal Experimental Endocarditis with Combinations of Cell Wall–Active Agents

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The efficacy of treatment with a combination of ampicillin, imipenem, and vancomycin was compared with that of two-drug combinations or monotherapy in a model of experimental endocarditis using a strain of Enterococcus faecium with high-level resistance to vancomycin and moderate intrinsic resistance to ampicillin and imipenem. In vitro time-kill synergy studies demonstrated bactericidal synergistic activity only for the triple combination. In vivo, monotherapy with vancomycin was not effective. Treatment with either ampicillin or imipenem alone or in combination with vancomycin resulted in <4 log₁₀ reduction in colony-forming units (cfu) per gram of vegetation. The combination of ampicillin with imipenem was highly active (an additional 5 log₁₀ reduction in cfu per gram of vegetation compared with the most active single agent), but efficacy was not increased by the addition of vancomycin to ampicillin and imipenem. Therapy with the combination of ampicillin and imipenem may be effective for some strains of multidrug-resistant enterococcal infections.

In recent years, the frequency of enterococcal infections has increased, and enterococci are now the second most commonly reported nosocomial pathogen in some studies [1]. The ability of enterococci to rapidly acquire multidrug resistance and to disseminate resistance determinants through the exchange of genetic elements [2] has resulted in serious therapeutic challenges [3]. The emergence of strains with high-level resistance to both gentamicin and streptomycin precludes bactericidal synergism between cell wall–active agents and these aminoglycosides [3–5]. Options for treatment of these serious enterococcal infections, such as administration of cell wall–active agents by continuous intravenous infusions, have limited efficacy [3, 6–8]. The isolation of strains with high-level resistance to glycopeptides and resistance to β-lactams further constrains treatment options [3, 9–12]. Moreover, these strains are often resistant to a variety of other antimicrobial agents and also may develop resistance to agents such as ciprofloxacin, novobiocin, or rifampin when used as monotherapy [12].

Several studies have demonstrated synergy in vitro with non-aminoglycoside-containing combinations, including daptomycin combined with fosfomycin [13], teicoplanin combined with imipenem [14], or ciprofloxacin combined with ampicillin [15], which may offer treatment options against selected highly vancomycin- and β-lactam–resistant enterococci. However, the activity in vivo of these combinations has not been determined in experimental animal models. Efficacy was demonstrated with either penicillin or ceftriaxone combined with vancomycin and gentamicin in experimental endocarditis due to highly vancomycin- and β-lactam–resistant enterococci [16–18]. Anecdotal reports suggested microbiologic cure of patients with highly vancomycin-resistant enterococcal bacteremia treated with ciprofloxacin combined with novobiocin [19] or ciprofloxacin combined with rifampin and gentamicin [20], even though the latter regimen was not effective in treatment of experimental endocarditis [21]. The efficacy of these treatment regimens, however, has been demonstrated only with strains that were not highly gentamicin- and ciprofloxacin-resistant.

Recent in vitro studies in one of our laboratories (C.W.S.) demonstrated bactericidal activity in vitro with the combination of subinhibitory concentrations of ampicillin, imipenem, and vancomycin against several multidrug-resistant enterococci (unpublished data). These studies suggested that combinations with these agents may be useful in vivo. The purpose of this study was to evaluate the efficacy of treatment of multidrug-resistant enterococcal experimental endocarditis using monotherapy or combinations of ampicillin, imipenem, and vancomycin.

Materials and Methods

Antimicrobial agents. Ampicillin sodium salt and vancomycin hydrochloride (Sigma, St. Louis) and imipenem crystalline (Merck...
Sharp & Dohme, Rahway, NJ) were used for in vitro testing. Pharmaceutical preparations of ampicillin used in vivo were purchased from Wyeth Laboratories (Philadelphia) and preparations of vancomycin were from Eli Lilly (Indianapolis). Imipenem 500-cilastatin for in vivo studies was provided by Merck Sharp & Dohme.

**Organisms.** For in vivo studies, an isolate of *Enterococcus faecium* was selected from a collection of enterococci with high-level resistance to vancomycin and non-β-lactamase-mediated resistance to penicillin and imipenem. Additionally, killing in vitro of this strain was enhanced by the combination of ampicillin, imipenem, and vancomycin compared with no antimicrobial or the best single agent.

**In vitro susceptibility testing.** A macrodilution method was used for testing the MIC with an inoculum of $5 \times 10^5$ cfu of enterococci/mL [22]. The MBC was defined as the lowest dilution that resulted at 24 h in a $\geq 3 \log_{10}$ decrease in the starting inoculum (99.9% killing) [23]. Testing for β-lactamase production in vitro was done with the nitrocefin disk test (Becton Dickinson, Cockeysville, MD).

**In vitro tests for synergy.** Time-kill curves were performed using an inoculum of $10^3-10^5$ cfu/mL in 25 mL of Mueller-Hinton broth. To prepare the inoculum, a mid-logarithmic phase growth culture was used [23, 24]. Cultures contained no antimicrobial agent (control) or subinhibitory concentrations of either a single antimicrobial or a combination of two or all three antimicrobials. After 0, 4, and 24 h of incubation at 35°C in room air, samples were removed from each culture and diluted serially; samples of each dilution were spread over the surface of a sheep blood agar plate to determine the number of colony-forming units (cfu) per milliliter. Synergy or antagonism was defined as a $\geq 100$-fold increase or decrease, respectively, in killing after incubation with the combination compared with the most active single agent. Indifference was defined as a $< 10$-fold increase or decrease in killing after incubation with the combination compared with the most active single agent [24]. Bactericidal synergism was defined as a reduction of the initial bacterial concentration 1000-fold below the initial inoculum [3, 24].

**In vivo studies.** Catheter-associated aortic valve experimental infective endocarditis was established in New Zealand White rabbits (weight, >2.5 kg) by a modification of the method described by Garrison and Freedman [25] as described [6]. Twenty-four hours after catheter placement, rabbits were infected by intravenous injection of $5 \times 10^6$ cfu/mL enterococci. Antimicrobial therapy was initiated 24 h after infection and continued for 3 days. Rabbits that died before administration of the first dose of therapy were excluded from analysis. The antimicrobial dosages (milligrams per kilogram of body weight) used were chosen to result in 30-min drug concentrations in serum of rabbits similar to those reported in humans receiving recommended therapeutic doses [26]. For each antimicrobial agent, concentrations in serum were assayed 30 min after administration of the first dose on the second day of treatment in the respective monotherapeutic treatment groups. A bioassay technique was used for all antimicrobial assays [27].

Rabbits were assigned randomly to a treatment group as follows (each group except controls had 16 rabbits): controls (11 rabbits), no treatment; ampicillin, 100 mg/kg, intramuscularly three times daily; imipenem, 60 mg/kg, intravenously three times daily; vancomycin, 15 mg/kg, intravenously twice daily; ampicillin plus vancomycin as above; vancomycin plus imipenem as above; ampicillin plus imipenem as above; ampicillin plus imipenem plus vancomycin as above.

Surviving animals were sacrificed by intravenous injection of 65 mg/kg sodium pentobarbital (Fort Dodge Laboratories, Fort Dodge, IA) at least 12 h after administration of the last dose of antimicrobial agents. At sacrifice, the chest was opened, the heart was excised and opened, and aortic valve vegetations were removed aseptically. Vegetations were weighed, homogenized with a Stomacher (Seward Laboratories, London), and cultured quantitatively using a pour plate method. Results were expressed as $\log_{10}$ cfu of enterococci per gram of valve vegetation. For purposes of quantitative nonparametric analysis, samples with no growth on the lowest dilution ("sterile" vegetations) were assigned half of the value of the lowest detectable quantity of microorganisms among all such samples ($\sim 0.6 \log_{10}$ cfu/g of vegetation).

**Statistical analysis.** The overall null hypothesis that no differences existed between any of the treatment groups was analyzed statistically with the Kruskal-Wallis test to estimate the per-experiment type I error rate. Individual pairwise comparisons were made only if the preliminary Kruskal-Wallis test indicated significant differences between treatment groups at the $\alpha = .05$ level [28]. Pairwise comparisons between treatment groups were made with the Wilcoxon rank sum test. The reported $P$ values for individual treatment group comparisons therefore reflect the comparisonwise type I statistical error rate conditional on an experimentwise error rate of $P \leq .05$ [28].

**Results**

**In vitro studies.** The MICs and MBCs for the strain used in vivo were, respectively, 16 and 64 μg of ampicillin/mL, 32 and 64 μg of imipenem/mL, and 512 and >512 μg of vancomycin/mL. High-level resistance to vancomycin and concomitant resistance to teicoplanin (MIC, 32 μg/mL) of the strain studied in vivo is consistent with a VVanA phenotype of vancomycin resistance [10]. The nitrocefin disk test did not demonstrate penicillinase production. Results of time-kill studies are shown in figure 1. While imipenem combined with vancomycin did not demonstrate synergy in vitro, the combination of ampicillin with either imipenem or vancomycin was synergistically inhibitory. The triple combination of ampicillin, imipenem, and vancomycin was the only regimen tested that was bactericidal in vitro.

**In vivo studies.** The concentrations in serum of antimicrobials measured 30 min after administration of the first dose on the second day of treatment were as follows: 90.0 mg of ampicillin/L, 76.5 mg of imipenem/L, and 44.4 mg of vancomycin/L.

Table 1 shows the results of treatment of experimental endocarditis. Preliminary overall analysis revealed a significant difference ($P < .01$) between treatment groups. Monotherapy with vancomycin was not different ($P = .13$) from no treatment. After 3 days of treatment, all other treatment regimens significantly ($P < .02$) reduced the number of bacteria in cardiac
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valve vegetations compared with no treatment. Monotherapy with imipenem was as effective ($P = .07$) as monotherapy with ampicillin, and both regimens were significantly more effective ($P < .01$) than was monotherapy with vancomycin. Therapy with the combination of ampicillin and imipenem was significantly ($P < .02$) more effective than was treatment with any single- or other double-drug regimen. There was no significant difference ($P = .93$) in the in vivo efficacy of triple-drug therapy with ampicillin, imipenem, and vancomycin compared with the outcome of treatment with the combination of ampicillin and imipenem. Treatment with the combination of imipenem and vancomycin was significantly more effective ($P < .01$) than was therapy with ampicillin alone or with the combination of ampicillin and vancomycin.

### Discussion

The lack of efficacy of monotherapy with vancomycin in our study was consistent with the high MIC of vancomycin, which exceeded the 30-min concentration of vancomycin in serum. The outcome of monotherapy with vancomycin in our study was consistent with previous studies that used vancomycin alone in highly vancomycin-resistant enterococcal experimental endocarditis [16]. Further, in our study, vancomycin therapy did not improve outcome when combined with either ampicillin or imipenem alone or with the combination of ampicillin and imipenem. These data support previous findings of Caron and colleagues [16-18], who failed to demonstrate an increased bactericidal activity of penicillin in combination with vancomycin in a model of highly vancomycin- and β-lactam-resistant experimental enterococcal endocarditis. These investigators suggested that the reported phenomenon of penicillin hypersusceptibility and penicillin-vancomycin synergy with vancomycin-resistant enterococci [29, 30] may occur only in vitro.

In contrast to that of vancomycin, the moderately resistant MICs of ampicillin or imipenem were within clinically achievable serum concentrations of both agents, and this effect was expressed in vivo with the results of monotherapy with either agent. Monotherapy with either ampicillin or imipenem was significantly more effective than was no therapy. Results of treatment with these agents are consistent with previous findings in studies of high-dose penicillin in moderately penicillin-resistant experimental endocarditis [16] but were less effective than results that represent effective therapy of enterococcal experimental endocarditis [31, 32].

The synergy observed in vitro for the combination of ampicillin and imipenem corresponded to the activity in vivo. Therm...
apy with the combination of ampicillin and imipenem was highly active and was the only regimen synergistic in vivo, although the study strain was resistant to both agents in vitro, and monotherapy with either agent resulted in only a minimal reduction in colony counts in cardiac valve vegetations. To determine the underlying mechanism of the marked synergy in vivo between ampicillin and imipenem against \( \beta \)-lactam-resistant enterococci, the critical target that mediates resistance to imipenem in enterococci needs to be defined. Synergy between ampicillin and imipenem against \( \beta \)-lactam-resistant enterococci may be associated with the saturation of different binding sites when resistance to imipenem is due to production or over-production of single or multiple penicillin-binding proteins different from those mediating resistance against penicillins in enterococci [33–35].

Treatment of serious multidrug-resistant enterococcal infections for individual patients must be based on in vitro susceptibilities of single or multidrug combinations. Currently there is no evidence for benefit from aminoglycoside- or quinolone-containing regimens against highly glycopeptide- and \( \beta \)-lactam-resistant strains that are concomitantly resistant to aminoglycosides or quinolones. The effectiveness of therapy with a combination of ampicillin and imipenem against these otherwise untreatable multidrug-resistant enterococci may offer a valuable treatment option for infections caused by these strains. Our data were generated from only a single strain of \textit{E. faecium} with moderate intrinsic resistance to \( \beta \)-lactams, and our findings should not be generalized to other multidrug-resistant enterococci. High-level glycopeptide-resistant strains of enterococci that are also highly resistant to \( \beta \)-lactams (MIC, \( >128 \mu g/mL \)) have been identified [9, 10, 14, 15, 21], and evidence suggests that levels of penicillin resistance have increased over time, primarily with \textit{E. faecium} [36], with MIC\textsubscript{50} and MIC\textsubscript{50} of 256 and 512 \( \mu g/mL \), respectively, in one study [37]. Further studies are needed to demonstrate the degree of correlation of bactericidal synergistic activity in vivo with in vitro synergy with the combination of ampicillin and imipenem against strains with higher level \( \beta \)-lactam resistance.

References

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