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Antibiotic Treatment of Experimental Endocarditis Due to Vancomycin- and Ampicillin-Resistant *Enterococcus faecium*

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We compared ciprofloxacin, rifampin, and gentamicin treatments, alone and in combination, for 5 days in the therapy of experimental aortic valve endocarditis in rats caused by a clinical isolate of vancomycin-resistant *Enterococcus faecium*. The MICs and MBCs of vancomycin, ciprofloxacin, rifampin, and gentamicin were 250 and >1,000, 3.1 and 6.3, 0.098 and 1.6, and 12.5 and >50 μg/ml, respectively. Infected rats were sacrificed after completing 5 days of therapy. Additional rats within each treatment group were followed for 5 days beyond the last dose of antibiotic therapy. Although survivals in the different groups were not significantly different after 5 days of therapy, survival was significantly better 5 days beyond the last dose of antibiotic therapy in rats treated with rifampin-containing regimens. The combination of ciprofloxacin and gentamicin was bactericidal in vitro and in vegetations from rats with enterococcal endocarditis. Rifampin alone was similarly bactericidal in vivo, but it was not significantly better than rifampin in combination with other antibiotics. Subpopulations resistant to rifampin, but not ciprofloxacin, were detected in the inoculum and in most vegetations during therapy. However, the combination of ciprofloxacin plus both gentamicin and rifampin reduced both the rifampin-susceptible and -resistant population in vegetations of 9 of 10 animals below the level of detection after 5 days of therapy. Nevertheless, a residual enterococcal population apparently remained in numbers of <2 $\log_{10}$ CFU/g after 5 days of therapy, which resulted in relapse. Perhaps a longer course of therapy would have eliminated this residual population and improved efficacy.

An aminoglycoside in combination with either penicillin or vancomycin has been the standard regimen for the treatment of serious enterococcal infections such as endocarditis (2). Recently, high-level penicillin resistance (MIC, >100 μg/ml) has been described in *Enterococcus faecium* (3, 21) and β-lactamase production has been described in *Enterococcus faecalis* (6, 13). High-level aminoglycoside resistance (MIC, >1,000 μg/ml) has been described in both species. Vancomycin-resistant enterococci have continued to be reported (8, 10, 17) with increasing frequency as a cause of nosocomial infections since they were first described by Uttley et al. in 1988 (19). Consequently, treatment of serious enterococcal infections has become problematic. We identified a nosocomial outbreak caused by a vancomycin-resistant *E. faecium* isolate that was also resistant to penicillin and streptomycin; in vitro data suggested that ciprofloxacin in combination with rifampin and gentamicin was active against this isolate (11). Bacteremic infections in several patients responded clinically and bacteriologically to this antibiotic combination. The purpose of the study described here was to determine the efficacies of ciprofloxacin, rifampin, and gentamicin, each alone and in combination, in a rat model of experimental endocarditis caused by this strain. We also evaluated the effect of the drug combination on the frequency at which resistance to ciprofloxacin or rifampin emerged during therapy.

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MATERIALS AND METHODS

In vitro microbiologic studies. The strain of vancomycin-resistant *E. faecium* used in the present study was isolated from a patient with bacteremia. Stock cultures were prepared by incubating the organism in cation-supplemented Mueller Hinton II broth (MHB; BBL, Baltimore, Md.) for 18 h at 37°C and freezing 1-ml samples at −70°C. For each experiment, a frozen sample was subcultured into MHB, and the mixture was incubated overnight at 37°C. The MICs and MBCs of vancomycin, ampicillin, gentamicin, rifampin (Sigma Chemical Co., St. Louis, Mo.), and ciprofloxacin (Miles, Inc., West Haven, Conn.) were determined by broth macrodilution methods by using MHB and an inoculum of 5×10^5 to 1×10^6 CFU/ml (15). To test for high-level resistance to streptomycin, 5×10^5 to 1×10^6 CFU of the test strain per ml was incubated in the presence of 2,000 μg of streptomycin (Sigma Chemical Co.) per ml. β-Lactamase production was tested by using a nitrocefin disk (Cefinase; BBL). Time-kill studies were performed by incubating the test isolate in 25-ml flasks of MHB or 50% rat serum in MHB that contained ciprofloxacin (3 μg/ml), gentamicin (3 μg/ml), rifampin (3 μg/ml), and various combinations of these agents (5, 18). An inoculum of an organism in the logarithmic phase of growth was added to each flask to achieve a final concentration of 5×10^5 CFU/ml. The flasks were mixed and then incubated in a shaking water bath at 37°C. Samples were removed at 0, 3, 6, and 18 h, and the numbers of CFU per milliliter in the flasks were determined by serial dilution and plating techniques. All in vitro susceptibility tests and time-kill studies were performed at least twice. The effects of antibiotic carryover were excluded by diluting the subculture below the MIC for the organism.

Therapeutic studies. Male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Altamount, N.Y.) weighing 200 to 300 g were anesthetized, and the right carotid artery of each

animal was cannulated. The arterial catheter was advanced across the aortic valve and secured subcutaneously (16). Twenty-four hours later, each rat was inoculated with 10^8 CFU of the vancomycin-resistant *E. faecium* in 1 ml of MHB via the tail vein. Eighteen hours after inoculation, the rats were divided into an untreated control group and the following six treatment groups: (i) gentamicin, 4 mg/kg of body weight administered intramuscularly every 12 h; (ii) ciprofloxacin, 40 mg/kg intramuscularly every 6 h; (iii) rifampin, 10 mg/kg intramuscularly every 12 h; (iv) ciprofloxacin plus gentamicin; (v) ciprofloxacin plus rifampin; or (vi) ciprofloxacin plus rifampin and gentamicin. Each treatment regimen was administered for 5 days. Survivors were sacrificed on the sixth day, at the time of trough levels in serum after the last dose of antibiotic, i.e., 6 h for ciprofloxacin and 12 h for rifampin and gentamicin. To assess the animals for relapse of infection, additional rats in each treatment group were followed for a 5-day period after the last dose of antibiotic therapy prior to sacrifice. Groups of untreated rats were sacrificed after 18 h, 5 days, and 10 days of infection.

At the time of sacrifice, vegetations on the aortic valve from individual rats were excised, pooled, and weighed. Vegetations from each animal were homogenized in 0.5 ml of MHB; the number of CFU per gram of vegetation was determined by standard serial dilution and plating techniques (4). Because of the small sizes of the vegetations (about 10 to 50 mg), the assay could not detect <2 $\log_{10}$ CFU/g. For sterile vegetations, the number of CFU was recorded as <2 $\log_{10}$ CFU/g of vegetation, but for statistical purposes they were assigned a value of 2 $\log_{10}$ CFU/g. In homogenates of vegetations that yielded a maximum of 1 to 29 colonies, the number of CFU per gram was also recorded as 2 $\log_{10}$ CFU/g for statistical purposes.

Detection of antibiotic resistance. The presence of antibiotic-resistant enterococci (mutant subpopulations) in the challenge inoculum and infected vegetations was detected by subculturing serial 10-fold dilutions of 10^9 CFU of the inoculum in the stationary growth phase or an homogenate of the vegetation on the surface of Mueller-Hinton agar that contained 10 times the MIC of rifampin or ciprofloxacin. The plates were incubated at 37°C for 72 h. The frequency of resistant mutants was calculated as the number of resistant colonies on the antibiotic-containing agar divided by the number of CFU plated.

Concentrations of antimicrobial agents. Blood for determination of peak concentrations of antibiotics in serum was obtained from tail veins 1 h after administration of either ciprofloxacin, rifampin, or gentamicin on the third day of therapy. Blood for trough concentration determinations was obtained from the inferior vena cava at the time of sacrifice. To document that there was no significant carryover of antimicrobial agent from the vegetations to the agar plates, trough levels of the antimicrobial agents in vegetations were also determined. The concentrations of antimicrobial agents were determined by an agar disk diffusion method (1) by using *Klebsiella pneumoniae* as the indicator organism for ciprofloxacin and *Bacillus subtilis* as the indicator organism for rifampin and gentamicin. The assay could not detect ciprofloxacin, rifampin, and gentamicin at concentrations of <0.08, <0.63, and <0.31 μg/ml, respectively, in serum and <1.1, <9.0, and <4.5 μg/g, respectively, in vegetations.

Statistical analysis. To determine significant differences in bacterial counts of the vegetations, a one-way analysis of variance and then the Tukey-A post hoc procedure was used, in which type I error for the entire set of pairwise comparisons was constrained to the reported *P* value. The chi-square test was used to determine the significance of differences in mortality.

TABLE 1. Antibiotic susceptibilities of the vancomycin-resistant strain of *E. faecium*

Antibiotic	MIC (μg/ml)	MBC (μg/ml)
Ciprofloxacin	3.1	6.3
Rifampin	0.1	1.6
Gentamicin	12.5	>50
Vancomycin	500	>1,000
Ampicillin	250	>1,000
Streptomycin	>2,000	

RESULTS

In vitro microbiologic studies. The MICs and MBCs of the antibiotics for the vancomycin-resistant *E. faecium* isolate are shown in Table 1. Figure 1 shows the rates of decrease in the numbers of *E. faecium* per ml of MHB in the time-kill studies. Use of 50% rat serum in MHB gave identical results (5). None of the antimicrobial agents used alone was bactericidal, but the combinations of ciprofloxacin plus either gentamicin, rifampin, or both gentamicin and rifampin resulted in a bactericidal effect at 18 h. Growth at 18 h in rifampin at 3 μg/ml was due to the emergence of a rifampin-resistant mutant (MIC, >50 μg/ml).

Therapeutic studies. The survival rate ranged from 63 to 100% at the end of 5 days of treatment and was not significantly different among the groups ($P > 0.05$) (Table 2). However, in rats held for an additional 5 days after the last dose of antimicrobial therapy, the survival rate was significantly better in the groups treated with rifampin-containing regimens (93%) than in either the untreated group (40%) or the group treated with a ciprofloxacin regimen not containing rifampin (42%) (chi-square [two degrees of freedom], 16.011; $P < 0.00034$; for rifampin-treated versus untreated rats, chi-square [one degree of freedom], 12.386, $P < 0.0005$ [Bonferoni corrected $P < 0.0015$]; for rifampin- versus ciprofloxacin-treated rats, chi-square [one degree of freedom], 13.095; $P < 0.003$ [Bonferoni corrected $P < 0.0009$]).

No sterile vegetations (<2 $\log_{10}$ CFU/g) occurred in the untreated group or the groups treated with gentamicin, ciprofloxacin, or ciprofloxacin plus gentamicin after 5 days of therapy. However, 18 of 31 rats receiving rifampin-

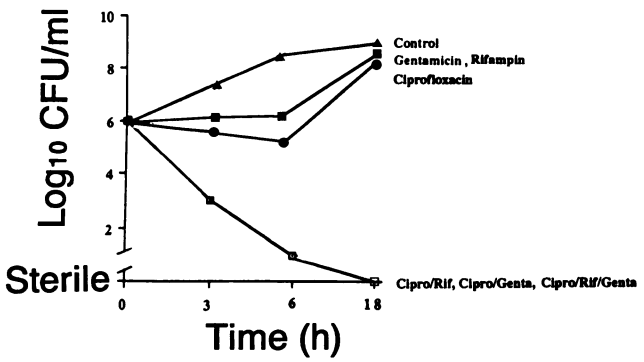

FIG. 1. Time-kill study of the vancomycin-resistant strain of *E. faecium* in ciprofloxacin (Cipro), rifampin (Rif), or gentamicin (Genta), each alone (3 μg/ml) or in combination. Sterile is <1.4 $\log_{10}$ CFU/ml.

TABLE 2. Frequency of survival of rats with experimental endocarditis caused by vancomycin-resistant *E. faecium* after 5 days of therapy and 5 days after the last dose of antibiotic therapy

Antibiotic regimen	No. of survivors/no. infected	
	5 days of treatment	5 days after last dose
Untreated	16/17	4/10
Gentamicin	5/8	
Ciprofloxacin	9/10	3/8
Ciprofloxacin plus gentamicin	9/10	4/8
Rifampin	10/10	8/9
Ciprofloxacin plus rifampin	11/11	10/10
Ciprofloxacin plus rifampin and gentamicin	10/10	8/9

containing regimens had sterile vegetations. In fact, 9 of 10 rats receiving ciprofloxacin, gentamicin, and rifampin had sterile vegetations at the end of 5 days of therapy.

The mean ± 1 standard deviation bacterial count in vegetations of untreated rats after 18 h of infection was 7.4 ± 0.5 $\log_{10}$ CFU/g. There was no significant difference in bacterial counts in vegetations among the untreated group (8.5 ± 1.3 $\log_{10}$ CFU/g) and groups treated with gentamicin (8.6 ± 1.4 $\log_{10}$ CFU/g) or ciprofloxacin (7.3 ± 1.8 $\log_{10}$ CFU/g) after 5 days of therapy ($P > 0.05$) (Fig. 2). Mean vegetation counts were significantly lower in groups treated with ciprofloxacin plus gentamicin (4.8 ± 1.8 $\log_{10}$ CFU/g), rifampin (3.4 ± 1.9 $\log_{10}$ CFU/g), ciprofloxacin plus rifampin (2.6 ± 1.2 $\log_{10}$ CFU/g), and ciprofloxacin plus gentamicin and rifampin (2.2 ± 0.2 $\log_{10}$ CFU/g) after 5 days of therapy than in the untreated group ($P < 0.01$) or groups treated with gentamicin ($P < 0.01$) or ciprofloxacin ($P < 0.01$) alone. The effectiveness of the combination of ciprofloxacin plus gentamicin was not significantly different from that of rifampin alone, but the combination was significantly less effective than the other two rifampin-containing regimens in reducing mean vegetation counts ($P < 0.05$). Mean vegetation counts were not significantly different among groups treated with rifampin, ciprofloxacin plus rifampin, or ciprofloxacin plus both gentamicin and rifampin ($P > 0.05$).

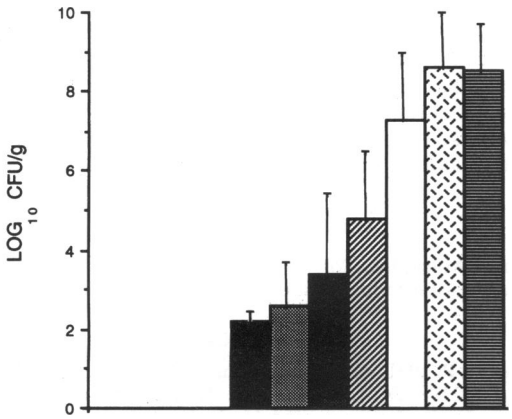

FIG. 2. Mean ± standard deviation enterococcal counts in vegetations after 5 days of no therapy or 5 days of therapy with various antimicrobial regimens. Bars represent data for rats treated with ciprofloxacin plus rifampin and gentamicin, ciprofloxacin plus rifampin, rifampin, ciprofloxacin plus gentamicin, ciprofloxacin, and gentamicin and untreated rats from left to right, respectively.

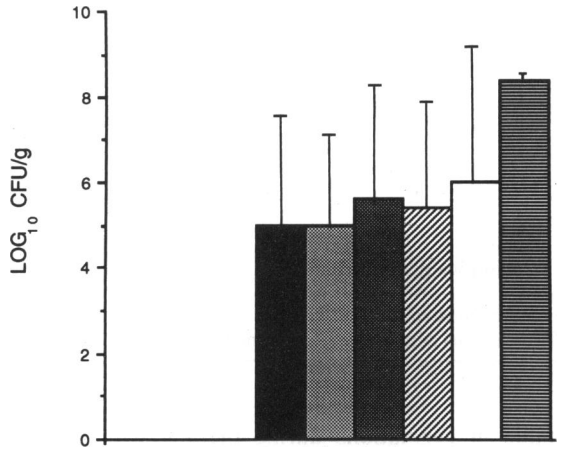

FIG. 3. Mean ± standard deviation enterococcal counts in vegetations after 10 days of no therapy or 5 days after the last dose of various antimicrobial regimens. Bars represent data for rats treated with ciprofloxacin plus rifampin and gentamicin, ciprofloxacin plus rifampin, rifampin, ciprofloxacin plus gentamicin, and ciprofloxacin and untreated rats from left to right, respectively.

This therapeutic efficacy was lost in rats held for an additional 5 days after the last dose of antimicrobial therapy. In surviving animals, mean vegetation bacterial counts did not differ significantly among the untreated group (8.4 ± 0.3 $\log_{10}$ CFU/g) and groups treated with ciprofloxacin (6.0 ± 3.2 $\log_{10}$ CFU/g), rifampin (5.6 ± 2.6 $\log_{10}$ CFU/g), ciprofloxacin plus gentamicin (5.4 ± 2.5 $\log_{10}$ CFU/g), ciprofloxacin plus rifampin (5.0 ± 2.1 $\log_{10}$ CFU/g), and ciprofloxacin plus both gentamicin and rifampin (5.0 ± 2.6 $\log_{10}$ CFU/g) at 5 days after the last dose of antibiotic therapy (Fig. 3).

Antimicrobial concentrations. The mean peak and trough concentrations of ciprofloxacin, rifampin, and gentamicin in serum were 4.1, 7.6, and 9.1 μg/ml and 1.2, 2.4, and <0.3 μg/ml, respectively. The mean trough concentrations of ciprofloxacin, rifampin, and gentamicin in vegetations were 1.8, <9.0, and <4.5 μg/g, respectively. At these levels, no significant carryover of antibiotic to the agar plates used for culturing the vegetations could have occurred.

Development of resistance. The frequency of spontaneous mutational resistance in the inoculum to 10 times the MIC was 4×10^{-5} for rifampin and $<1 \times 10^{-9}$ for ciprofloxacin. The frequencies at which spontaneous mutational resistance to 10 times the MIC of rifampin and ciprofloxacin occurred in vegetations of untreated controls were 5×10^{-4} and $<1 \times 10^{-7}$, respectively. *E. faecium* resistant to rifampin at 10 times the MIC was also detected in the vegetations of rats from each of the treatment regimens after 5 days of therapy but not in rats treated with ciprofloxacin plus gentamicin and rifampin (Table 3). Rifampin-resistant isolates remained in low numbers (2 $\log_{10}$ CFU/g) or were a minor portion of the total enterococcal vegetation counts except in one rat treated with rifampin alone for 5 days, in which the total population of 7.5 $\log_{10}$ CFU of enterococci per g was resistant to 10 times the MIC. At 5 days beyond the last dose of antibiotic therapy, rifampin-resistant subpopulations were detected in many of the animals treated with ciprofloxacin plus rifampin, ciprofloxacin plus gentamicin, and ciprofloxacin plus both rifampin and gentamicin, but the rifampin-resistant population remained in low numbers or was a minor portion of the total enterococcal population.

Ciprofloxacin resistance to 10 times the MIC was not

TABLE 3. Development of rifampin resistance in vegetations of infected rats

Antibiotic regimen	No. of rats with rifampin-resistant enterococci/no. of infected rats studied	
	5 days of therapy	5 days after last dose
Untreated	4/5	0/2
Ciprofloxacin		0/3
Ciprofloxacin plus gentamicin	2/5	2/3
Rifampin	1/5	0/4
Ciprofloxacin plus rifampin	5/6	6/6
Ciprofloxacin plus rifampin and gentamicin	0/5	2/4

detectable in vegetations during therapy with any of the ciprofloxacin-containing regimens. However, *E. faecium* isolates resistant to ciprofloxacin were detected in vegetations from six of six rats 5 days after the last dose of the combination of ciprofloxacin plus rifampin. In five of these rats, the count of the ciprofloxacin-resistant population was at the lowest limit of detection (2 $\log_{10}$ CFU/g), with a mean total enterococcal count of 5.8 $\log_{10}$ CFU/g, and in one of these rats, the count of the resistant population was 5.0 $\log_{10}$ CFU/g, with a mean total enterococcal count of 7.9 $\log_{10}$ CFU/g.

DISCUSSION

As worldwide reports of enterococcal resistance to standard antimicrobial agents increase, therapeutic options have dramatically diminished. The fluoroquinolones have demonstrated in vitro activity against enterococci; however, the MICs are usually close to the peak concentrations in serum, e.g., 3 to 4 μg of ciprofloxacin per ml (14). In addition, data supporting the use of these agents for the treatment of serious enterococcal infections are limited. The ciprofloxacin MIC of 3.1 μg/ml for our isolate was similar to values reported for enterococci by others (9, 14). Similarly, rifampin has not been thought to be an effective agent against enterococci, alone or in combination with other antibiotics, despite MICs of ≤16 μg/ml (12). Nevertheless, we demonstrated that ciprofloxacin plus either gentamicin, rifampin, or the combination of rifampin plus gentamicin is bactericidal in vitro against this particular strain.

The rat model of experimental endocarditis provided in vivo data that support these in vitro observations. Survival was improved at 5 days after the last dose of antibiotic in rats treated with rifampin-containing regimens. While vegetations from rats treated with ciprofloxacin or gentamicin alone had bacterial counts similar to those for untreated controls, the combination of ciprofloxacin plus gentamicin caused a bactericidal effect in vivo. Rifampin alone caused a bactericidal effect similar to that of ciprofloxacin plus gentamicin, and there was no therapeutic difference among the various rifampin-containing regimens. The therapeutic effect could not be attributed to enhancement of antibiotic activity by rat serum, because this strain failed to show in vitro such an effect that has been shown with other strains of enterococci (5).

Since resistance emerges rapidly when rifampin is used alone against enterococci, this drug is not a therapeutic option as a single agent (12). In fact, we isolated a rifampin-resistant subpopulation in the initial inoculum and in vegetations from many of the rats treated for 5 days with all of the regimens except with the combination of ciprofloxacin plus both gentamicin and rifampin. Vegetations from 9 of 10 rats treated with ciprofloxacin plus both gentamicin and rifampin were sterile (<2 $\log_{10}$ CFU/g) at the end of therapy. Nevertheless, at 5 days beyond the last dose of antibiotic therapy, a predominantly rifampin-susceptible population with a rifampin-resistant subpopulation was recovered from the vegetations of rats in all treatment groups.

The reasons why the rifampin-resistant isolates usually did not become predominant remain speculative. Since we tested for rifampin resistance at only 10 times the MIC (1.0 μg/ml), it is possible that the concentration of rifampin in vegetations (<9 μg/g) was sufficient to inhibit, but not kill, the rifampin-resistant enterococci during therapy with rifampin. We also noted that growth of rifampin-resistant organisms on rifampin-containing agar was always slow (20), and this slow growth possibly could account for the smaller populations of rifampin-resistant organisms in vegetations during rifampin therapy. However, these explanations could not account for the presence of rifampin-resistant subpopulations in the vegetations of untreated rats, rats treated with non-rifampin-containing regimens, or rats at 5 days after the last dose of antibiotic therapy.

We identified ciprofloxacin-resistant isolates in only two rats, which were treated for 5 days with ciprofloxacin and rifampin and sacrificed 5 days after the last dose. The emergence of ciprofloxacin resistance during therapy has been reported with other bacterial species, and rifampin has been reported to decrease the emergence of resistance to ciprofloxacin in a study of experimental endocarditis caused by *Staphylococcus aureus* (7, 22). However, ciprofloxacin resistance was evaluated at only 10 times the MIC. Lower levels of resistance might have occurred since resistance to this drug may occur in a stepwise fashion.

These data show that when ciprofloxacin and gentamicin are used in combination, a synergistic bactericidal effect is seen both in vitro and in vegetations from rats with enterococcal endocarditis. Rifampin alone is bactericidal in vivo, but the efficacy of this drug is limited by a relatively high rate of spontaneous mutation to rifampin resistance. In addition, the efficacy of rifampin is strain dependent; rifampin, alone or in combination, was reported to be of no therapeutic value when a less susceptible strain was studied (6). The combination of ciprofloxacin and gentamicin plus rifampin reduced both the rifampin-susceptible and -resistant populations in vegetations below the level of detection. However, a residual enterococcal population apparently remained in numbers that were less than the lower limit of detection (<2 $\log_{10}$ CFU/g) after 5 days of therapy and that resulted in relapse. Perhaps a longer course of therapy would have eliminated this residual population and improved the treatment's efficacy.

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