ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1992, p. 121–125 Vol. 36, No. 1
0066-4804/92/010121-05$02.00/0
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Antimicrobial Susceptibility Changes in *Enterococcus faecalis* Following Various Penicillin Exposure Regimens

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Received 13 April 1991/Accepted 22 October 1991

Penicillin-"virgin" strains of *Enterococcus faecalis* collected from a population of individuals with no previous antibiotic exposure were subjected in vitro to penicillin delivered as repeated pulses, stepwise increasing concentrations, or sustained levels of a single concentration. Changes in resistance to penicillin were assessed by determination of MICs, and time-kill studies were performed to evaluate changes in tolerance to the bactericidal effects of penicillin. Isogenic clones, derived from various exposure regimens, which exhibited changes in either resistance or tolerance were further examined for changes in penicillin-binding proteins. Exposure to repeated pulses of penicillin resulted in the development of tolerance to penicillin without changes in the level of resistance. Clones derived from a regimen of stepwise increases in the penicillin concentration acquired both increased penicillin resistance and tolerance. Clones selected after prolonged continuous exposure to a fixed concentration of penicillin displayed minimally increased resistance to penicillin, but they retained the lytic, nontolerant response to the bactericidal effect of penicillin. Clones which acquired tolerance to the bactericidal effect of penicillin without changes in penicillin resistance exhibited a penicillin-binding protein pattern identical to that of the parental strain. Increased labeling of several penicillin-binding proteins accompanied the development of increased penicillin resistance in both penicillin-tolerant and nontolerant strains. Exposure of *E. faecalis* to penicillin in repeated pulses of brief duration, for prolonged periods at a constant concentration, or in stepwise graded concentrations can result in the selection of clones with increased resistance to the inhibitory or bactericidal effects of penicillin, or both. These observations may be relevant to the selection of dosing regimens for penicillin in the treatment of enterococcal infections, when bactericidal synergism cannot be achieved with penicillin-aminoglycoside combinations.

Serious infections with *Enterococcus faecalis* pose a significant therapeutic challenge since a unique combination of resistance characteristics allows it to survive in the presence of many antibiotics. Strains of *E. faecalis* exhibit low-level resistance to aminoglycosides (3) and to lincomycin and clindamycin (10); they exhibit an intermediate level of resistance to penicillins and cephalosporins (15). In addition, most clinical isolates exhibit tolerance to the bactericidal effects of cell wall-active agents (7, 12). Alarming trends in recent years include the emergence of strains with high-level resistance to aminglycosides (11), the appearance of β-lactamase-producing isolates (14), and the discovery of strains that are resistant to vancomycin (19).

Serious enterococcal infections have traditionally been treated with combinations of a penicillin and an aminoglycoside which result in bactericidal synergism. However, the widespread emergence of high-level aminoglycoside resistance in this species has rendered the usual combinations ineffective against some isolates, and optimal treatment for infections caused by highly gentamicin-resistant strains has not been established. In an earlier study which explored the effectiveness of ampicillin alone in an animal model of enterococcal endocarditis (18), we found an apparent advantage of continuous intravenous infusion compared with intermittent intramuscular injection of the same total daily dose, even when probenecid was given with the latter. While continuous-infusion regimens have been used clinically, recent practice prescribes the use of most antimicrobial therapy as discrete pulses. At this time it is not known whether continuous-infusion regimens may offer some therapeutic advantage in human infections. In addition, previous work in our laboratory suggested that occasional enterococcal isolates are not tolerant to the bactericidal effects of penicillin and that the level of penicillin resistance or tolerance could be affected by penicillin exposure in vitro (22). To determine the effect of different penicillin exposure regimens on the tolerance and resistance of enterococci, we exposed cultures of antibiotic-"virgin" *E. faecalis* to continuous, pulsed, or stepwise increasing concentrations of penicillin.

MATERIALS AND METHODS

Bacterial strains. The *E. faecalis* strains used in these studies included organisms collected over 20 years ago from the feces of a primitive, isolated human population of the highlands of Malaita, Solomon Islands. This population had no previous antibiotic exposure, and therefore, the strains represent penicillin-virgin strains from the preantibiotic era (5). Of these strains, SI-E39 was used for the experiments described below. SI-E39 exhibits a nontolerant, lytic response to penicillin and penicillin susceptibility similar to that of current U.S. clinical isolates (MIC, 3.1 μg/ml). Strain E1 is a clinical isolate of *E. faecalis* isolated from a patient in Boston with endocarditis. Strain E1 is inhibited by penicillin at 4 μg/ml, exhibits a bacteriostatic response to penicillin, and has been described in detail elsewhere (13).

In preliminary experiments in our laboratory, when SI-E39 was serially passed on plates containing increasing concentrations of penicillin, a series of isogenic strains with

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stepwise increases in penicillin resistance was obtained, and it was demonstrated that the increased penicillin resistance was accompanied by penicillin-binding protein (PBP) changes that include a parallel increase in the density of PBPs 1, 4, and 5 (21). The penicillin MIC ranged from 6.2 to 31 μg/ml for the clones that were selected in this manner.

Media. *E. faecalis* was grown in glucose (dextrose) phosphate broth (Scott Laboratories, Inc., West Warwick, R.I.) supplemented with 1% sodium citrate (DPBc) (17). Glucose phosphate agar was prepared from DPBc with 1.7% Bacto Agar (Difco Laboratories, Detroit, Mich.).

Reagents. Benzyl[^{3}H]penicillin ethylpiperidinium salt (11.874 μCi/ml) was generously supplied by Merck Sharp & Dohme Research Laboratories, Rahway, N.J. Unlabeled benzylpenicillin was obtained from Eli Lilly & Co., Indianapolis, Ind. Chemicals of electrophoretic grade purity for preparation of polyacrylamide gels were obtained from Bio-Rad Laboratories, Richmond, Calif. 2,5-Diphenyloxazole was purchased from New England Nuclear Corp., Boston, Mass. Penicillinase was obtained from BBL, Division of Beckman, Dickinson, and Co., Cockeysville, Md. Other reagents were purchased from various commercial sources.

MICs. The level of resistance to penicillin was expressed in terms of the MIC, which was determined by standard techniques of broth and agar dilution (16). Broth dilutions were accomplished with serial twofold dilutions of penicillin prepared in DPBc medium, with a final tube receiving no antibiotic, which served as a control for growth. Each tube was then inoculated with a mid-logrithmic-phase culture, yielding a final inoculum of approximately 10^5 CFU/ml, and was incubated overnight at 35°C. The MIC was the lowest concentration of antibiotic which prevented visible growth. To determine susceptibility by agar dilutions, bacterial suspensions were prepared from fresh blood agar plates and applied with a multiprong inoculating device to yield a final inoculum of approximately 10^4 CFU per spot.

Growth and time-kill studies. Tolerance to killing by penicillin was determined by time-kill techniques. Although we did not arbitrarily set criteria by which to define tolerance prior to the start of our study, it became evident that some strains exhibited virtually no killing on exposure to penicillin (<1-$\log_{10}$-unit decrease in CFU/ml), whereas others, with some variation between experiments, always demonstrated at least a 1,000-fold reduction in viable organisms. We refer to such strains as tolerant and nontolerant, respectively. Overnight both cultures were diluted 1:100 into five fresh, prewarmed tubes of DPBc, and their growth was monitored by following the A_{660} with a Sequoia-Turner spectrophotometer. At an A_{660} of 0.200 (1×10^8 to 2×10^8 CFU/ml), different concentrations of penicillin were added to four of the tubes (MIC, 5× the MIC, 10× the MIC, or 100× the MIC), while the fifth tube was used to monitor growth in the absence of antibiotic. Viability was determined by removing 0.5-ml aliquots at various times. These were treated with penicillinase, diluted into saline, and plated onto antibiotic-free blood agar. The dilution and plating method used for colony counts had as a minimum level of detection 20 to 200 CFU/ml, depending on whether direct samples (prior to the first dilution) were taken. Because of incomplete killing of even the nontolerant strains, actual colony counts were always at least 10-fold higher than this highest minimum level of detection. Growth and lysis were followed spectrophotometrically.

PBPs. Since alterations of PBPs have been shown to accompany increased beta-lactam resistance in enterococci (1, 4), PBPs were assayed as follows. Log-phase cultures harvested at an A_{660} of 0.200 were concentrated 10-fold in fresh DPBc, and aliquots of 0.1 ml were incubated for 60 min at 37°C with [^{3}H]penicillin at concentrations equal to the MIC, 10× the MIC, and 100× the MIC (21). The reaction was stopped by the addition of excess unlabeled penicillin and placement in an ice bath. Cells were recovered by centrifugation (10 min, 4,000 × *g*) and lysed by incubating them for 25 min at 37°C in potassium phosphate buffer (10 mM, pH 6.8) containing lysozyme (0.1 mg/ml), M1 muramidase (0.1 mg/ml), and 0.1% (wt/vol) Triton X-100, with Sarkosyl added to a final concentration of 0.006% during the last 5 min of incubation. Membrane proteins, including labeled PBPs, were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were stained with Coomassie blue; destained with methanol, acetic acid, and water; and then processed, dried, and exposed to prefogged XAR-2 Kodak film for 4 days at −70°C.

Penicillin exposure regimens. (i) Pulsed-exposure regimen. The pulsed-exposure regimen simulated exposure of organisms to peak and trough concentrations of penicillin obtained by intermittent administration to patients. Organisms were exposed to brief pulses of penicillin at 10× the MIC and were then allowed to grow in drug-free medium. This cycle was repeated five times.

To accomplish this, an overnight broth culture was diluted 1:100 into fresh DPBc and incubated at 37°C. Growth was followed spectrophotometrically, and viability was determined at 1-h intervals. At an A_{660} of 0.200, the culture was divided into two equal parts, and 10× the MIC of penicillin was added to one-half of the culture, while the other half received no drug and served as a control. Both cultures were incubated at 37°C for 7.5 h, a time of exposure that has been determined to cause 2- to 3-log-unit viability loss in strain SI-E39 exposed to penicillin at this concentration. The cells were then harvested by centrifugation, washed three times in antibiotic-free medium, and grown overnight in DPBc. Penicillin exposure was repeated in this manner five times, and on the sixth exposure solid medium was used to allow selection of the surviving clones as follows. An overnight culture of the fifth pulsed culture in broth was diluted 1:100 and was allowed to grow to an A_{660} of 0.200. At that time, several aliquots were plated onto DPAc plates containing 10× the MIC of penicillin. Half of the plates were incubated for 7.5 h at 37°C, while the other half of the plates were allowed to grow for 24 h at 37°C. After these times, the plates were sprayed with penicillinase and incubated overnight. Survivors were selected, purified, and characterized by MIC, time-kill studies, and PBP analysis.

(ii) Stepwise graded concentration regimen. With the stepwise graded concentration regimen, an attempt was made to select penicillin-resistant colonies by serial passage of strains on progressively increasing concentrations of penicillin. Specifically, colonies from an overnight blood agar plate were streaked onto DPAc containing penicillin at its MIC against the organism. Colonies which grew on the MIC plates were selected and plated onto media containing 2× the MIC of penicillin, and the surviving clones were subsequently selected and plated through a series of plates containing twofold increases in the penicillin concentration. From each pass, clones were selected, purified, and subjected to susceptibility tests, time-kill studies, and PBP studies.

(iii) Prolonged constant concentration regimen. The prolonged constant concentration regimen was designed to reproduce in the laboratory the continuous infusion of beta-lactam antibiotics considered to be a potential thera-

peutic strategy in serious enterococcal infections caused by high-level gentamicin-resistant organisms. By this method, organisms were maintained for 5 days in culture medium that contained a fixed concentration of penicillin at or above the MIC.

To accomplish this, overnight cultures were diluted 1:100 in fresh DPBc and allowed to grow at 37°C to an A_{660} of 0.200. This early-log-phase culture was then divided into 10-ml aliquots; and penicillin was added at 0.5× the MIC, MIC, 5× the MIC, and 10× the MIC. After 8 h, cultures were diluted (0.2 ml into 9.8 ml) in DPBc containing identical penicillin concentrations. Cultures were maintained at 37°C for 5 days by diluting them into fresh medium containing the same penicillin concentration twice daily. At the end of each day, viability was determined by combining an aliquot from each tube with excess penicillinase, diluting it in saline, plating it onto antibiotic-free blood agar, and counting the colonies after overnight incubation at 35°C. The surviving clones were isolated, purified, and characterized by MIC, time-kill curves, and PBP studies.

Confirmation that constant levels of penicillin were present in the media throughout the period of continuous exposure was established by bioassay by using an agar well technique with *Bacillus subtilis* as the test organism (2). Over an 18-h period there was no more than 7.5% loss of penicillin in the medium.

RESULTS

Pulsed exposure regimens. Strain SI-E39 showed a nontolerant response to penicillin exposure, with viability decreases ranging from approximately 3.5 to 4.5 log CFU/ml at 24 h, accompanied by lysis with a decrease in the A_{660} by approximately 0.200 to 0.300 units/24 h. The reasons for the modest variability in killing observed in different experiments are not evident, but these results differed markedly from those with clinical strain E1, which did not lyse and which exhibited a bacteriostatic response to inhibitory concentrations of penicillin (data not shown). After exposure of the nontolerant, lytic strain SI-E39 to five pulses of penicillin at 31 μg/ml (10× the MIC), 2 of 10 clones selected after 7.5 h of incubation and 5 of 10 clones selected after 24 h of incubation on DPAc-penicillin plates were tolerant to penicillin. These clones retained the tolerant response to penicillin after subculture on antibiotic-free medium. Penicillin MICs against all the tolerant clones remained the same as that against the parental strain SI-E39 (3.1 μg/ml). Results of time-kill studies of four tolerant clones, T9, T15, T18, and T23, selected by penicillin pulsing, in comparison with time-kill studies of the parental strain SI-E39, are shown in Fig. 1. The clones selected by pulsed penicillin exposure exhibited a tolerant, bacteriostatic response to penicillin concentrations that were 10× the MIC, which was similar to the response of strain E1 and in sharp contrast to the nontolerant response of SI-E39. The PBP patterns of several tolerant clones appeared to be identical to that of SI-E39. Strain SI-E39 has six PBPs with molecular weights ranging from 43,000 to 115,000, a pattern that is similar to that of *E. faecalis* isolates described elsewhere, for which the MIC of penicillin is similar (6, 20).

Stepwise graded concentration regimens. Figure 2 demonstrates that the isogenic penicillin-resistant strains selected by exposure to stepwise graded concentrations of the drug acquired penicillin tolerance as well. MICs of penicillin against these strains ranged from 6.2 to 31 μg/ml.

Prolonged constant concentration regimens. After 5 days of continuous exposure of strain SI-E39 (nontolerant; penicillin MIC, 3.1 μg/ml) to 3.1 μg of penicillin per ml, 30 clones were isolated from the viability plates. MICs of penicillin against these clones were equal to those of the parenteral strain or at most twofold higher. One of these clones (E39-4; MIC, 6.2 μg/ml) was subjected to a repeat round of continuous penicillin exposure, and again, increases in the penicillin MIC resulted, as follows. Against 29 of 32 clones derived from E39-4 selected from the 10× MIC viability plate, penicillin MICs were 12.5 μg/ml; against 25 of 27 clones derived from the 5× MIC viability plate, penicillin MICs were 12.5 μg/ml. Results of time-kill studies of strains SI-E39 and E39-4 and two clones selected from continuous penicillin exposure of E39-4 (E39-4A2 and E39-4A3) are shown in Fig. 3. Like the parental strain SI-E39, and in marked contrast to the clones that were selected after both pulsing and exposure to increasing concentrations, the more resistant clones were nontolerant to penicillin, with killing and lysis occurring at

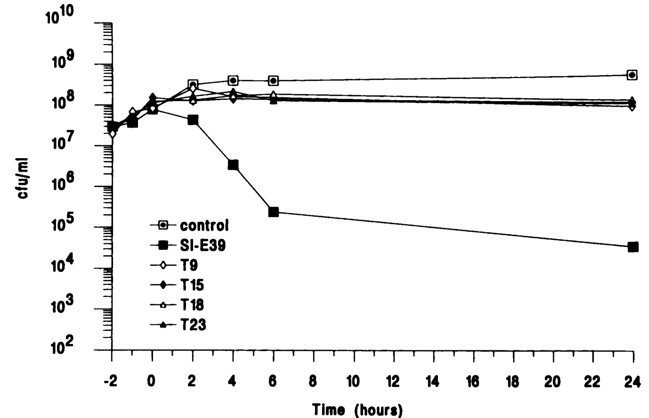

FIG. 1. Viability in the presence of penicillin of T9, T15, T18, and T23, four clones selected by penicillin pulsing, compared with that of parent strain SI-E39. For each strain, penicillin was added at 10× the MIC. SI-E39 grown in the absence of antibiotics is shown as a growth control.

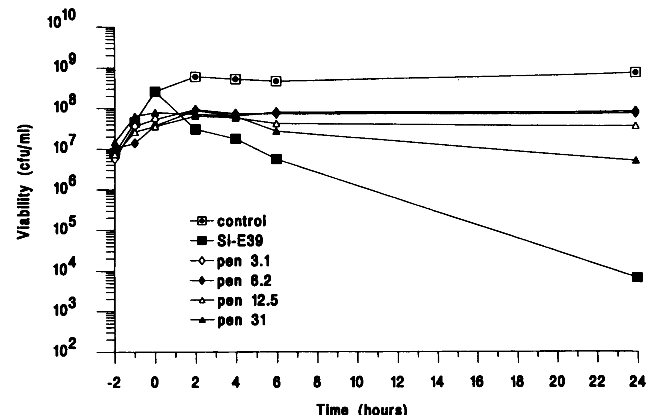

FIG. 2. Viability of parental strain SI-E39 and four clones (identified by penicillin MIC) which were selected by exposure to stepwise increases in penicillin concentration. Penicillin was added at 10× the MIC against each strain. SI-E39 grown in the absence of antibiotics is shown as a growth control.

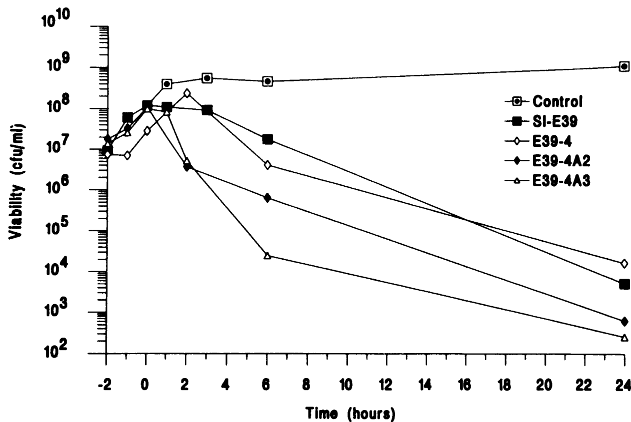

FIG. 3. Viability in the presence of penicillin of parental strain SI-E39 and three relatively penicillin-resistant clones selected from continuous penicillin exposure. For clone E39-4, the penicillin MIC was 6.2 μg/ml, while for clones E39-4A2 and E39-4A3, which were derived from an additional round of continuous penicillin exposure, MICs were 12.5 μg/ml. Penicillin was added at 10× the MIC determined against each strain. An an antibiotic-free control SI-E39 is shown.

concentrations of penicillin that were adequate to inhibit growth (9).

Clones derived from continuous penicillin exposure of T23 (penicillin tolerant; penicillin MIC, 3.1 μg/ml; derived by pulse exposure; see above and Fig. 1) were selected on day 5 from the MIC viability plates. Again, a twofold increase in the MIC was seen against all 30 clones tested. Continuous exposure of two of these clones (T23-1 and T23-2) again resulted in further increases in resistance. For 26 of 29 clones selected at day 5 from the 0.5× MIC viability plates, penicillin MICs were 25 μg/ml, and for two clones, MICs of penicillin were 12.5 μg/ml. Figure 4 shows results of time-kill studies of T23, T23-2, T23-2/2, and T23-2/17; the last two strains were selected after continuous exposure of T23-2 to penicillin. Exposure of these strains, all of which were

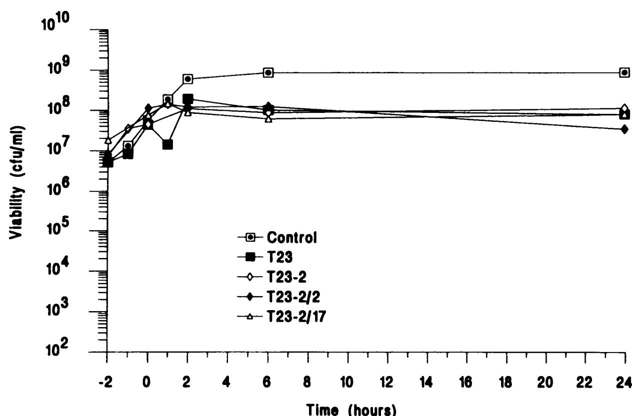

FIG. 4 Viability of T23, a tolerant clone derived from SI-E39 by penicillin pulsing, and three relatively penicillin resistant clones selected from continuous penicillin exposure of T23. Penicillin MICs for T23-2, T23-2/17, and T23-2/2 were 6.2, 12.5, and 25 μg/ml, respectively. Penicillin was added at 10× the MIC. SI-E39 grown in the absence of antibiotic served as a growth control.

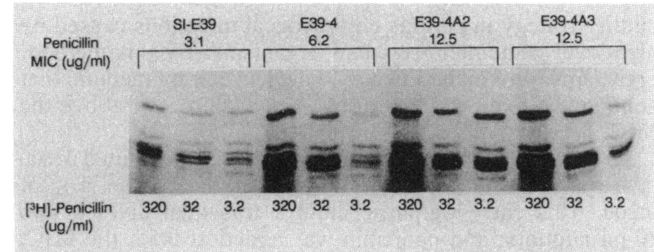

FIG. 5. PBPs of three nontolerant, penicillin-resistant clones (E39-4, E39-4A2, and E39-4A3) compared with those of parental strain SI-E39.

derived from a tolerant clone, to inhibitory concentrations of penicillin did not result in a significant loss of viability.

The PBPs of some of the more resistant, nontolerant clones selected by continuous penicillin exposure compared with those of the parental strains are shown in Fig. 5. Increased labeling of PBPs 1, 4, and 5 were seen in the more resistant clones E39-4, E39-4A2, and E39-4A3. These changes were identical to those seen previously in relatively penicillin-resistant, tolerant clones selected by subjecting the same parental strain (SI-E39) to stepwise increasing concentrations of penicillin (21).

DISCUSSION

The facts that the susceptibilities to penicillin (MIC) and the PBP patterns of the pre-antibiotic-era strains from the Solomon Islands are similar to those of current clinical isolates of *E. faecalis* suggest that this particular complement of PBPs is responsible for the low-level resistance to beta-lactams universally associated with *E. faecalis*. No differences in PBPs were noted when isogenic strains differing only in tolerance characteristics were compared.

The current treatment of enterococcal infections typically involves the delivery of antibiotic doses at time intervals that result in peak and trough concentrations in serum. Results of the experiments described here indicate that this pulsed exposure to penicillin does not select for clones for which the MICs are increased, and it may explain why the level of penicillin resistance in clinical isolates of *E. faecalis* is comparable to that of antibiotic-virgin strains, despite the extensive use of beta-lactams in the clinical setting. It is interesting to speculate that before the introduction of antibiotics all strains of *E. faecalis* may have been nontolerant, like the Solomon Island isolates, and that the clinical use of penicillins administered as discrete doses or pulses selected for tolerance without affecting the level of penicillin resistance (MIC).

In contrast, increases in penicillin resistance accompanied by changes in PBPs were obtained in the laboratory when SI-E39 was exposed to stepwise increasing concentrations of penicillin or, to a lesser extent, when they were exposed to unchanging concentrations for a prolonged time. However, the former regimen led also to the development of tolerance, while the latter regimen did not. Thus, the response of bacteria to antibiotic pressure may be at least partially dependent on the method of antibiotic administration.

We have not yet explored the molecular mechanisms by which the organisms develop tolerance of and resistance to lysis by penicillin; these mechanisms probably involve either changes in the autolytic enzymes or their regulatory system or, perhaps, changes in cell wall composition that result in

diminished susceptibility to disruption by the autolytic system. Why the penicillin exposure regimen should result in selection of tolerant or resistant clones in some cases but not in others remains completely unclear.

The frequent occurrence of high-level gentamicin resistance and the resultant resistance to synergistic killing by combinations of penicillins and aminoglycosides have resulted in instances of serious enterococcal infections for which treatment of proven efficacy is not available. Experiments that involve exposure to constant concentrations of penicillin closely mimic in the laboratory the conditions of continuous infusion of ampicillin; these conditions have been used experimentally (8, 18). On the basis of successful treatment of enterococcal endocarditis in rats with continuous ampicillin infusion (8), similar regimens have been suggested as possibly being effective in some cases of serious human enterococcal infections caused by organisms which are highly resistant to aminoglycosides. Our results presented here appear to indicate that continuous-infusion regimens may favor the selection of strains with modestly increased beta-lactam resistance, although in these experiments penicillin MICs against the more resistant clones remained within clinically achievable levels. However, the experimental conditions of repeated dilution into fresh penicillin-containing DPBc may allow more growth of organisms than that which occurs in cardiac vegetations, thus artificially favoring the emergence of resistant organisms. More important, perhaps, is the evidence that continuous exposure did not select for penicillin tolerance. Whether or not continuous-infusion regimens would offer a unique advantage to individual patients against particular isolates of *E. faecalis* with various degrees of penicillin resistance and tolerance remains to be determined.

ACKNOWLEDGEMENT

This work was supported in part by a grant from Eli Lilly & Co.

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