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In Vitro Response to Bactericidal Activity of Cell Wall-Active Antibiotics Does Not Support the General Opinion that Enterococci Are Naturally Tolerant to These Antibiotics

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The incidence of tolerance and paradoxical response to bactericidal activity of penicillin was investigated in 50 clinical isolates of *Enterococcus faecalis*. Of the isolates tested, 86% exhibited the paradoxical phenomenon whereby there were more survivors at high than at low concentrations above the MIC. Low penicillin concentrations caused decreases equal to or higher than 99.9% in 11 strains, from 99.9 to 99.5% in 23 strains, and lower than 99.5% in 9 strains. Of the total strains, 14% were killed to the same extent by all concentrations above the MIC. The bactericidal activities of other beta-lactams (ampicillin and piperacillin) and other cell wall inhibitors (vancomycin and daptomycin) were also tested against some of these strains. In general, beta-lactams exhibited the best bactericidal activity at 2× MIC. Piperacillin was the most active, as at 2× MIC it reduced the original inoculum by 99.9% or more in most of the strains. No concentration of vancomycin above the MIC caused 99.9% killing of the strains, whereas daptomycin was bactericidal at 8× MIC in most cases. Paradoxical response to bactericidal activity of beta-lactams was abolished by incubation of the inoculum with 2× MIC before exposure to higher antibiotic concentrations. These findings suggest that enterococci are not always tolerant to cell wall-active antibiotics and that accurate in vitro bactericidal tests may be useful for the choice of appropriate therapy for infections caused by these microorganisms.

Tolerance of bacteria to antibiotics is defined as delayed or decreased bacterial killing by growth-inhibiting concentrations of bactericidal compounds. For clinical purposes, a strain is defined as tolerant when the MIC/MBC ratio is ≥32 (the MBC is the minimal concentration causing 99.9% killing of the inoculum [5, 13]).

Enterococci, mainly *Enterococcus faecalis*, are susceptible to some cell wall inhibitors but were considered to be naturally tolerant to these antibiotics (6, 9–11, 16). However, clinical isolates of this species often exhibited a peculiar type of tolerance to cell wall inhibitors, in particular, to some beta-lactams, in that these bacteria may be killed by relatively low antibiotic concentrations (2× to 4× MIC) but the percentage of survivors increases at increasing antibiotic concentrations (paradoxical effect) (8, 14). From the clinical point of view, this phenomenon might pose less therapeutical problems than true tolerance, as the bactericidal concentrations can be theoretically achieved in body tissues.

In previous studies, we have shown that paradoxical response to penicillin is an intrinsic and stable property of a strain which is expressed by cultures of all ages, even if it is better expressed by cultures in the late log and stationary phases of growth (3).

Since enterococci are susceptible to very few antibiotics, in this study, we investigated whether or not in vitro differentiation between true tolerance and paradoxical response is possible and whether or not this may provide a rational basis for not excluding potentially active and nontoxic drugs in the therapy of infections caused by enterococci. To make our bactericidal tests more sensitive and reliable, we used high-density inocula of stationary-phase cells instead of the standard low-density inocula of actively growing cells, as the former conditions are more suitable for detection of paradoxical response and tolerance (3, 13) and more closely reflect the physiological situation of bacteria in an infected body area.

MATERIALS AND METHODS

Strains. The *E. faecalis* strains used in these studies were clinical isolates obtained from different body areas. These isolates were initially identified as enterococci by growth in 6.5% salt broth and by hydrolysis of esculin in the presence of 40% bile (2). Subsequent identification was performed with the API-Strep system (API-System S.A. Analytab Products). All experiments were performed by growing bacteria in Mueller-Hinton broth (Difco Laboratories) containing 1.5% agar when used as solid medium (Mueller-Hinton agar).

Antibiotics and reagents. Penicillin and ampicillin were purchased from E. R. Squibb & Sons, piperacillin was from Lederle Laboratories, and vancomycin was from Lilly, Italy. All other chemicals were reagent grade commercially available products.

Bactericidal activity testing. Glass tubes containing serial twofold dilutions of antibiotic in 5 ml of Mueller-Hinton broth were inoculated with 50 μl of an overnight culture. The final density of bacteria per milliliter was determined immediately after dilution of the inoculum in the tube not containing the antibiotic, and in general, it was around 10^7 CFU/ml. After 20 h of incubation at 37°C, all tubes were vortexed, and 4 h later they were examined for visual turbidity and the MIC was recorded. After the tubes were vortexed again, a 0.1-ml sample was taken from each tube without visual turbidity and serially diluted and 0.1 ml of each dilution was spread onto two Mueller-Hinton agar plates (8-cm diameter). This procedure was necessary to obtain a countable number of colonies, in particular, from samples incubated with high antibiotic concentrations. CFUs were counted after 24 h of

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TABLE 1. Distribution of penicillin exposure survival percentages for PRP-positive and -negative strains in a total of 50 *E. faecalis* isolates

Strain phenotype	Total no.	No. of strains showing a survival %[a] of:					
		≤0.1	0.2	0.3	0.4	0.5	≥0.5
PRP positive	43	11	6	6	6	5	9
PRP negative	7	6					1

[a] The survival percentage is the fraction of the original inoculum which survived after incubation at 37°C for 24 h with the penicillin concentration giving the maximal bactericidal effect. This concentration was, in general around 2× to 4× MIC. The survival percentages were found to be similar when low-density inocula of actively growing cells were used as recommended for standard MBC determination (13).

incubation. For this study, a paradoxical bactericidal effect of penicillin was considered to be represented by a significant increase in counts of survivors from at least three consecutive tubes containing antibiotic concentrations above the MIC, and strains exhibiting such behavior were regarded as positive in terms of paradoxical response to penicillin (PRP-positive strains). In testing the bactericidal activities of beta-lactams, antibiotic carryover was eliminated by adding penicillinase to the samples before plating. However, simple dilution of the samples was also sufficient to reduce the concentration of the antibiotic carried over to subinhibitory levels.

For killing curves, the procedure described for MIC determination was also used, but CFU counting was done at 0, 2, 4, 6 and 14 h of incubation at 37°C.

RESULTS

The incidence of paradoxical response and tolerance to the bactericidal activity of penicillin among *E. faecalis* clinical isolates was evaluated by using 50 strains which were susceptible to this antibiotic (MIC, ≤4 μg/ml) and by determining the number of surviving bacteria at concentrations ranging from 2× MIC to 32× MIC after 24 h of incubation at 37°C.

Of 50 strains, 43 (86%) were PRP positive (Table 1). In all of these strains, the minimal percentage of survivors was found at concentrations ranging from 2× to 8× MIC. Under these conditions, the decreases in the initial inoculum were equal to or higher than 99.9% for 11 strains (22% of the total strains), from 99.8 to 99.5% for 23 strains (46%), and lower than 99.5% for 9 strains (18%). At concentrations of >8× MIC, the percentage of surviving bacteria increased from 10- to 100-fold in all PRP-positive strains (data not shown).

Seven strains proved PRP negative, and six of these were 99.9% killed at all concentrations of ≥2× MIC (Table 1).

The bactericidal activities of several other antibiotics active on the cell wall (beta-lactams and non-beta-lactams) were then tested against six PRP-positive strains and one PRP-negative strain (strain 3678).

All strains were susceptible to all of the antibiotics tested (Table 2). All of the beta-lactams were, in general, more bactericidal at 2× MIC than at higher concentrations against all of the strains, but not against strain 3678. Piperacillin was the most active of the beta-lactams, and the decrease it caused in the initial inoculum was equal to or more than 99.9% at all concentrations equal to or above 2× MIC in most cases.

No vancomycin concentrations above the MIC were bactericidal against most strains, including strain 3678, whereas daptomycin was the only antibiotic which never caused the paradoxical effect and proved bactericidal against all strains.

TABLE 2. Antibacterial activities of several cell wall inhibitors against *E. faecalis* strains

Strain	Antibiotic[a]	MIC (μg/ml)	% of survivors after 24 h of incubation at:		
			2× MIC	8× MIC	32× MIC
E6	Pen	4	0.4	1.4	1.4
	Amp	2	0.04	0.16	1.2
	Pip	8	0.03	0.06	0.08
	Van	2	0.2	0.5	0.8
	Dap	4	0.1	0.003	0
258.3	Pen	2	0.6	2.3	11.5
	Amp	2	0.09	3.1	4.5
	Pip	2	0.09	0.001	1.8
	Van	2	1.3	2.2	1.4
	Dap	4	0.5	0.02	0.002
24	Pen	4	0.4	0.6	3.1
	Amp	1	0.4	0.3	8.5
	Pip	2	0.2	0.02	0.06
	Van	1	1.9	2.4	3.3
	Dap	4	0.7	0.05	0.001
263.61	Pen	4	0.6	2.6	12.3
	Amp	1	0.4	1	5.2
	Pip	8	0.04	0.009	0.1
	Van	1	6.7	7	8.3
	Dap	4	1	0.006	0
263.1	Pen	4	0.5	0.8	3.3
	Amp	1	0.2	2.5	3.1
	Pip	8	0.001	0.001	0.01
	Van	1	3.8	2.9	5
	Dap	4	1.7	0.4	0.003
21	Pen	4	0.6	1	3.5
	Amp	1	0.8	5.7	6.8
	Pip	8	0.001	0.01	0.1
	Van	1	0.3	0.3	0.4
	Dap	4	1.4	0.04	0.01
3678	Pen	4	0.2	0.2	0.1
	Amp	1	0.3	0.3	0.3
	Pip	4	0.1	0.001	0.001
	Van	1	1	1	1
	Dap	4	0.1	0.005	0

[a] Pen, Penicillin; Amp, ampicillin; Pip, piperacillin; Van, vancomycin; Dap, daptomycin.

Time-kill experiments with ampicillin, piperacillin, vancomycin, and daptomycin were performed with E6, a PRP-positive strain, and 3678, a PRP-negative strain. In this assay, too, piperacillin exhibited the best bactericidal activity against the PRP-positive strain and killed the enterococcal cultures with faster kinetics than did ampicillin (Fig. 1). With piperacillin, the rate of killing approached the maximal value as early as within the first 6 h of incubation.

Against strain 3678, ampicillin and piperacillin yielded similar killing kinetics at concentrations equal to and above 2× MIC (Fig. 2).

The time-kill experiments also confirmed the lack of any significant killing activity of vancomycin and the good bactericidal activity of daptomycin, which was the most active antibiotic in absolute terms, especially at the highest concentration used (Fig. 1 and 2).

In a previous report, we suggested that the paradoxical response of enterococci to penicillin could be a consequence of the rapid inhibition of peptidoglycan synthesis which

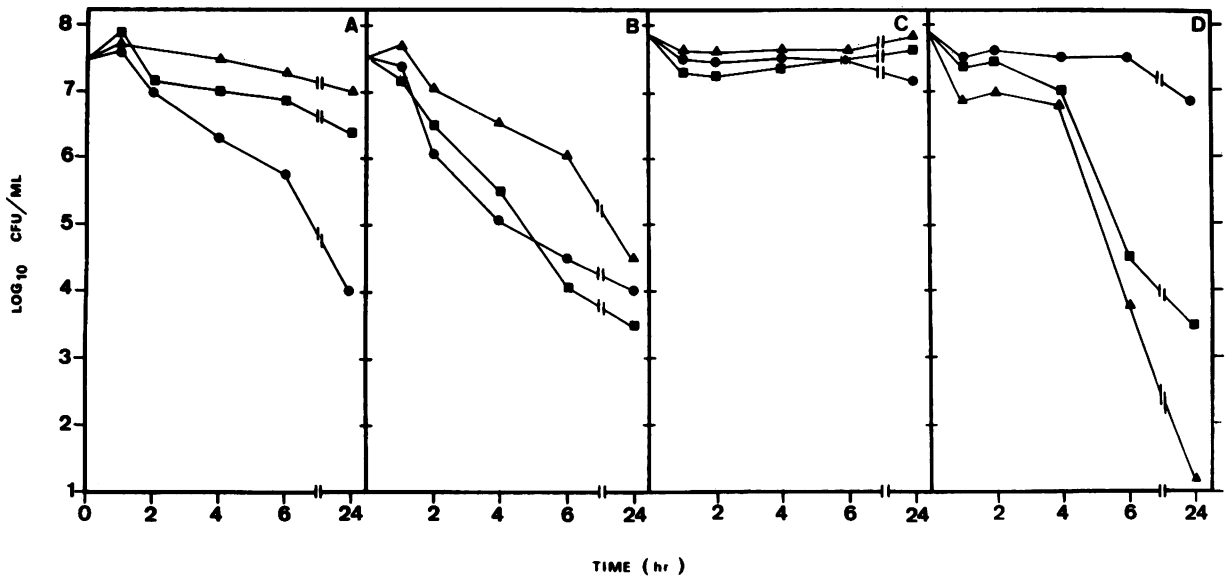

FIG. 1. Bactericidal activities of ampicillin (A), piperacillin (B), vancomycin (C), and daptomycin (D) against *E. faecalis* E6 at 2× (●), 8× (■), and 32× (▲) MIC.

occurs when bacteria which are in a particular phase of the cell cycle (the so-called phase of resistance to bactericidal activity) were exposed to high antibiotic concentrations (3). As a result, the percentage of survivors might be higher with faster exposure to high antibiotic levels.

To verify this hypothesis, we performed an experiment in which strains E6 and 3678 were first incubated for different periods with 2× MIC of ampicillin and then with a higher antibiotic concentration which was found to induce the paradoxical response. After 4 and 24 h of incubation at this latter concentration, the percentage of surviving bacteria was determined. Strain E6 exposed for 24 h to 128 μg/ml exhibited the paradoxical response to ampicillin but failed to do so when it was treated for at least 60 min with 8 μg/ml (2× MIC) before the antibiotic concentration was brought up to 128 μg/ml (Fig. 3A). Under the latter conditions, the rate of killing of the E6 strain culture was comparable to that of the culture incubated for 24 h with 8 μg/ml.

On the contrary, incubation of strain 3678 with 8 μg of ampicillin per ml did not significantly increase the susceptibility of this strain to the bactericidal activity of high antibiotic concentrations (Fig. 3B).

DISCUSSION

The results of this study showed that tolerance and paradoxical response to in vitro bactericidal activity of penicillin were two distinguishable phenomena which could

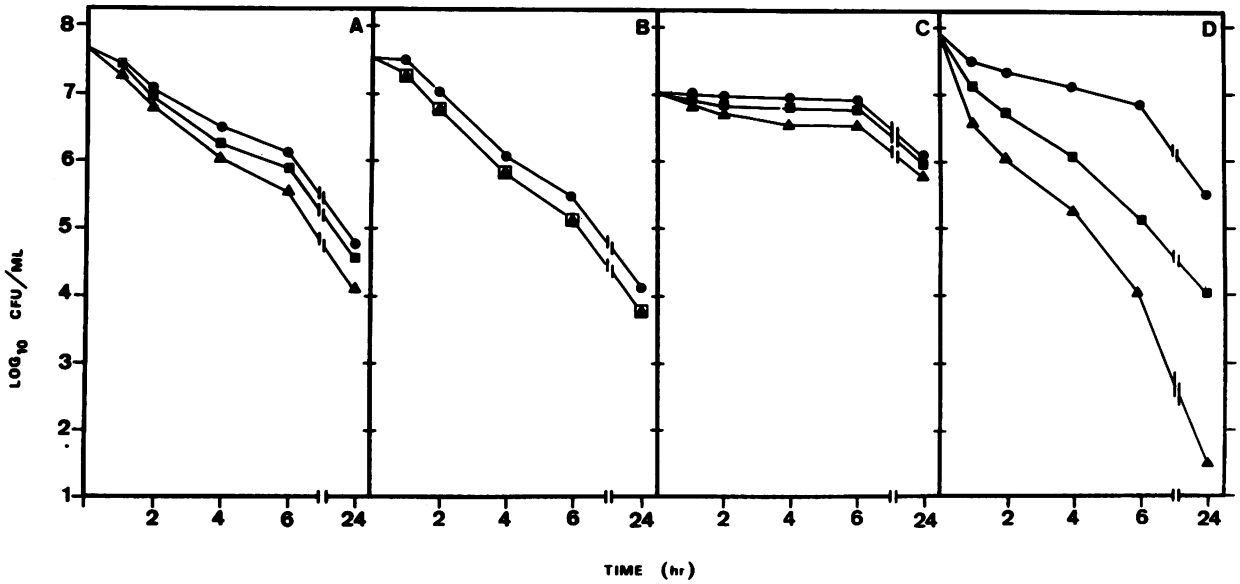

FIG. 2. Bactericidal activities of ampicillin (A), piperacillin (B), vancomycin (C), and daptomycin (D) against *E. faecalis* 3678 at 2× (●), 8× (■), and 32× (▲) MIC.

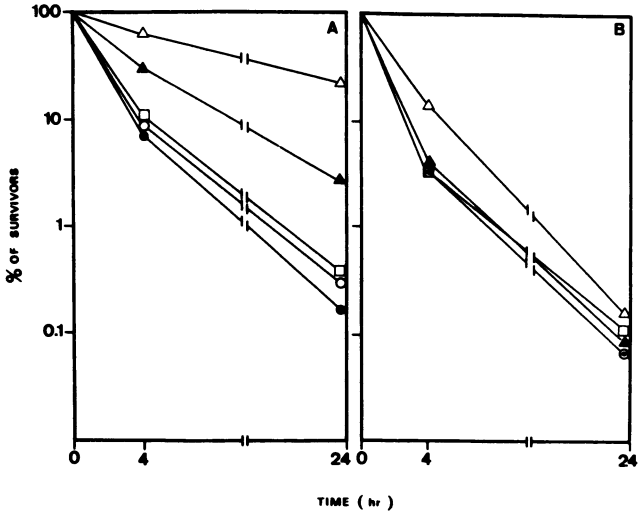

FIG. 3. Effect of exposure of *E. faecalis* E6 (A) and 3678 (B) cultures to 2× MIC of ampicillin on the bactericidal activity of a high concentration of the same antibiotic. Cells were exposed for 30 (▲), 60 (□), or 120 (○) min to 8 μg of ampicillin per ml, and then the antibiotic concentration was raised to 128 μg/ml. Control cultures were exposed to 8 (●) or 128 (△) μg/ml for 24 h.

occur simultaneously, separately, or not at all in *E. faecalis* strains. In fact, 22% of the strains could be considered paradoxically responding but not tolerant (as penicillin concentrations of 2× MIC caused a 99.9% reduction in initial inoculum); 65% were paradoxically responding and tolerant, as no antibiotic concentration above the MIC caused the stated 99.9% decrease in the initial inoculum; 12% were neither paradoxically responding nor tolerant; and only 2% were tolerant but not paradoxically responding. If, as stated also by others, we consider that a 1,000-fold reduction of the original inoculum is an arbitrary requirement and there is not much evidence that 99 or 98.0% would be a less useful definition (13), we may conclude that most *E. faecalis* strains may be efficiently killed by relatively low penicillin concentrations.

It should be stressed that these experiments were performed with high-density inocula of stationary-phase cells which allowed better phenotypical expression of both tolerance and paradoxical response (13) than would low-density inocula of actively growing cells. Under the former conditions, the incidence of these two phenomena might be overestimated but the results of the test might be more reliable and safe, as bacteria grow very slowly at infection sites and may give rise to high-density populations.

The additional bactericidal tests performed on seven strains with other beta-lactams (ampicillin and piperacillin) and cell wall inhibitors (vancomycin and daptomycin) showed that piperacillin and daptomycin exhibited a strong bactericidal activity against enterococci. Piperacillin caused a >99.9% reduction in the initial inoculum at 2× MIC in all strains. Although the percentage of survivors increased at higher concentrations, in nearly all cases it did not exceed 99.9%. Daptomycin was bactericidal at 8× and 32× MIC against all strains and never induced the paradoxical effect.

Although several studies besides ours have already shown that some penicillins and cephalosporins have bactericidal activity against *E. faecalis* (1, 4, 7, 12, 14, 15, 17, 18), tolerance to the bactericidal activity of cell wall inhibitors is still considered to be a natural feature of this species and a cause of failure of therapy of infection due to enterococcal strains which are susceptible to these antibiotics in vitro. As most strains which show a paradoxical response are efficiently killed by low antibiotic concentrations, the question of whether or not such resistance to the bactericidal activity of high antibiotic concentrations has any effect on the outcome of therapy arises. The finding (Fig. 3) that the paradoxical response was less evident or absent when bacteria were exposed for 30 to 60 min to low concentrations of ampicillin before the antibiotic concentration was increased suggested that the phenomenon has no clinical relevance, as bacteria in vivo are exposed to dynamic concentrations, or at most it would decrease the potential therapeutic efficacy of a beta-lactam only if it were given by using a therapeutic regimen which causes too rapid an increase in the drug concentration at the site of infection.

This hypothesis is supported by the in vivo studies of Thauvin et al. (15), who showed that in experimental enterococcal endocarditis, continuous-infusion therapy with ampicillin was significantly more effective than intramuscular injections in reducing bacterial titers in cardiac vegetations and that no statistically significant advantages were found for high-dose compared with low-dose ampicillin.

On the basis of our findings, we suggest that also in susceptibility tests against enterococci the MBC endpoint should be considered as the minimal concentration that gives at least a 99.9% decrease in the original inoculum, even if the number of survivors increases at higher concentrations. Of course, additional studies with in vivo experimental infections are required to assess whether infections caused by PRP-positive or -negative *E. faecalis* strains with an MBC/MIC ratio of <32 for a cell wall-active antibiotic may be cured by the antibiotic alone. However, the proposed criteria for interpretation of bactericidal tests against enterococci may provide a useful approach for identification of truly tolerant strains and comparison of various therapeutic regimens.

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