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Defective Killing of Enterococci: a Common Property of Antimicrobial Agents Acting on the Cell Wall

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We tested the ability of antimicrobial agents that act on the cell wall to kill enterococci and found defective killing (a minimal bactericidal concentration/minimal inhibitory concentration ratio of ≥32) with both β-lactams (penicillin G and cephalothin) and non-β-lactams (vancomycin, cycloserine, and bacitracin). Our results indicate that the resistance of enterococci to antimicrobial killing spans the spectrum of agents known to inhibit cell wall synthesis and suggest that the mechanism responsible for enterococcal resistance to killing by these drugs may be a defective autolytic enzyme system.

One of the many features that distinguish enterococci from other streptococci is their antibiotic resistance: enterococci are characteristically resistant to the bactericidal effects of penicillin (10) and cephalothin (23). Indeed, their resistance to β-lactam killing is the reason why bacteremic human enterococcal infections require synergistic (bactericidal) therapy with penicillin and an aminoglycoside (9, 11, 15, 22). Therefore, we have been especially interested in the mechanism of enterococcal resistance to β-lactam killing.

To determine whether this resistance was restricted to β-lactams, we examined the bacteriostatic and bactericidal effects of both β-lactams (penicillin G and cephalothin) and non-β-lactams that act on the bacterial cell wall (vancomycin, cycloserine, and bacitracin) against 34 clinical enterococcal isolates. The results of those studies form the basis of this report.

MATERIALS AND METHODS

Strains. The 34 enterococcal strains used in these studies were unique clinical isolates obtained from blood cultures in the Barnes Hospital Microbiology Laboratory between January 1977 and April 1979. After their initial isolation and identification from tryptic soy broth (Difco), these strains had been frozen at −70°C in brucella broth (Pfizer) with 15% glycerol. We thawed these cultures and subcultured them first on blood agar plates and then on nutrient agar slants (Remel), which were used to inoculate overnight cultures for both the identification and susceptibility studies. These isolates were initially identified as enterococci by growth in 6.5% salt broth and by the hydrolysis of esculin in the presence of 40% bile (4, 11). Subsequent identification was performed by the methods of Facklam (4) and Gross et al. (5).

We studied 10 viridans group streptococci and 9 *Streptococcus bovis* strains that had been isolated from blood cultures during the same time period and stored similarly at −70°C. We also tested four *S. bovis* isolates kindly provided by Robert C. Moellering, Jr. These strains had been initially identified by alpha hemolysis with negative tests for the hydrolysis of sodium hippurate (1) and esculin (viridans group streptococci) or by the hydrolysis of esculin in the presence of bile and failure to grow in 6.5% salt broth (*S. bovis*).

The group D precipitin reaction was determined with group D antiserum (Difco) by the method of Rantz and Randall (12), with group D antigen (Difco) as a positive control. We used 40-ml overnight cultures in Todd-Hewitt broth (Difco) and lysozyme treatment (5 mg/ml for 4 h at 37°C) (21) for bile-esculin-positive strains that failed to react with the group D antiserum after the Rantz and Randall procedure (one *S. bovis* isolate).

We used commercially prepared bile-esculin medium, 6.5% salt broth, arginine decarboxylase medium, and litmus milk (Remel). Potassium tellurite (Difco), 2,3,5-triphenyltetrazolium (Sigma) agar, 2% starch agar, and heart infusion broths for acid production from carbohydrates were prepared by the method of Facklam (4). Carbohydrates tested were D-sorbitol, D-mannitol, D-melibiose, L-arabinose (Sigma), and D-glycerol (Difco). Broth for acid production from sodium pyruvate (Sigma) was prepared as described by Gross et al. (5). Todd-Hewitt broth was used to grow overnight cultures for the identification tests, Mueller-Hinton broth (Difco) was used to grow the inocula for susceptibility testing, and sheep blood agar plates (Remel) were used to subculture tubes without visible growth in the tube dilution studies of antimicrobial susceptibility.

Antimicrobial agents. The antimicrobial agents used were penicillin G (Pfizer), cephalothin (Lilly), vancomycin (Lilly), cycloserine (Sigma), and bacitracin (Sigma).

Identification testing. A sterile Pasteur pipette was used to inoculate each test with 2 drops of an overnight culture in Todd-Hewitt broth; cultures were examined daily for 3 days. Tests and positive results were as follows: group D precipitin reaction, formation of a white cloud or ring in the capillary tube within 10

min; bile-esculin medium, obvious growth and production of a dark brown or black color; 6.5% salt broth, growth; litmus milk, change of the indicator from purple to white and clot production; tetrazolium, red colonies; potassium tellurite, dark black colonies; arginine hydrolysis, production of a purple color; starch hydrolysis, failure to stain after exposure to Gram iodine; acid production from carbohydrates, change of the indicator from purple to yellow (from green to yellow for sodium pyruvate).

Susceptibility testing. We determined the minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of these antimicrobial agents for enterococci by previously described methods (2, 20). Blood agar plates were inoculated from the nutrient agar slants and incubated overnight (all cultures were incubated at 37°C). On the next morning, tubes containing Mueller-Hinton broth were inoculated from the overnight cultures, incubated for 2 to 4 h, and diluted with saline to match a 0.5 McFarland no. 1 standard (20). These suspensions were diluted 1:200 (vol/vol) with plain Mueller-Hinton broth to approximately 2×10^5 colony-forming units per ml and mixed 1:1 with Mueller-Hinton broth containing twice the desired concentration of the antimicrobial agent to be tested. This produced the desired range of drug concentrations and a final inoculum of approximately 10^5 colony-forming units per ml.

The MIC was the lowest concentration of antimicrobial agent which prevented visible growth after 18 to 24 h of incubation. Tubes without visible growth were subcultured to blood agar plates (50 μl spread over half of a 100-mm plate). The MBC was the lowest concentration of antimicrobial agent which produced a ≥99.9% kill (i.e., five or fewer colonies from 50 μl) after 48 h of incubation.

Defective killing. We defined defective killing as an MBC/MIC ratio of ≥32; this is also the definition of penicillin tolerance used by Sabath for staphylococci (13).

RESULTS

Identification studies. The results of tests to differentiate *Streptococcus faecalis*, *Streptococcus faecium*, and *S. bovis* were generally in agreement with previous reports (4, 5, 11) (Table 1). The unexpected negative tests for tellurite resistance in *S. faecalis* (6 of 30 organisms tested) presumably reflected growth inhibition by the tellurite medium, since only 4% of the tests were positive by 24 h, as compared with the tetrazolium test, in which 100% of tests were positive at 24 h. Clot production in litmus milk by *S. faecium* strains was not associated with more rapid growth and remains unexplained. The 10 viridans group streptococci had negative tests for hippurate hydrolysis and for esculin hydrolysis in the presence of bile.

Susceptibility studies. Defective killing (an MBC/MIC ratio of ≥32) was a common property of the five antimicrobial agents tested against enterococci (*S. faecalis* and *S. faecium*) (Table 2), although the individual MICs and MBCs of the different drugs varied widely (Fig. 1). Surviving organisms showed no evidence of altered MICs or MBCs. Defective killing with these five agents was also observed with nonenterococcal group D streptococci (*S. bovis*) (Table 2). Because previous reports have found no significant differences between the MICs and MBCs of *S. bovis* (11), we retested the four *S. bovis* isolates. These organisms also demon-

TABLE 1. *Identification of group D streptococci*[a]

Test	*S. faecalis*		*S. faecium*		*S. bovis*	
	Expected	Observed	Expected	Observed	Expected	Observed
Group D reaction	+	30/30	+	4/4	+	9/9
Bile-esculin	+	30/30	+	4/4	+	9/9
6.5% Salt broth	+	30/30	+	4/4	−	0/9
Acid in litmus milk	+	30/30	+	4/4	+	9/9
Clot in litmus milk	+	26/30	−	4/4	+	9/9
Reduction of tetrazolium	+	30/30	−	0/4	V	0/9
Resistance to tellurite	+	24/30	−	0/4	−	0/9
Acid from pyruvate	+	30/30	−	0/4	−	0/9
Hydrolysis of arginine	+	30/30	+	4/4	−	0/9
Hydrolysis of starch	−	0/30	−	0/4	+	9/9
Acid from:						
Sorbitol	+	30/30	−	1/4	−	0/9
Mannitol	+	30/30	+	4/4	V	3/9
Arabinose	−	0/30	+	4/4	V	0/9
Melibiose	−	1/30	+	4/4	V	5/9
Glycerol	+	30/30	−	0/4	−	0/9

[a] The expected results are based on previous reports (4, 5, 11), and the observed results are expressed as the ratio of the number of positive tests to the number of isolates tested (30 *S. faecalis*, 4 *S. faecium*, and 9 *S. bovis* strains). Symbols for expected results are as follows: +, positive; −, negative; V, variable. See text for details of strain selection and test interpretation.

TABLE 2. *Defective killing of group D streptococci*[a]

Antimicrobial agent	Enterococci		*S. bovis*		Viridans group streptococci	
	MBC/MIC	Median MIC	MBC/MIC	Median MIC	MBC/MIC	Median MIC
Penicillin	128	2.0	256	0.03	2	0.015
Cephalothin	≥256	31.0	256	0.12	2	0.06
Bacitracin	32	125.0	64	31.0	4	8.0
Cycloserine	≥128	250.0	≥64	125.0	4	125.0
Vancomycin	≥16,000	0.5	512	0.5	8	0.25

[a] The MBC/MIC is expressed as the median MBC/MIC ratio of all isolates tested, the median MICs are expressed in micrograms per milliliter, and defective killing is defined as an MBC/MIC ratio of ≥32 (13).

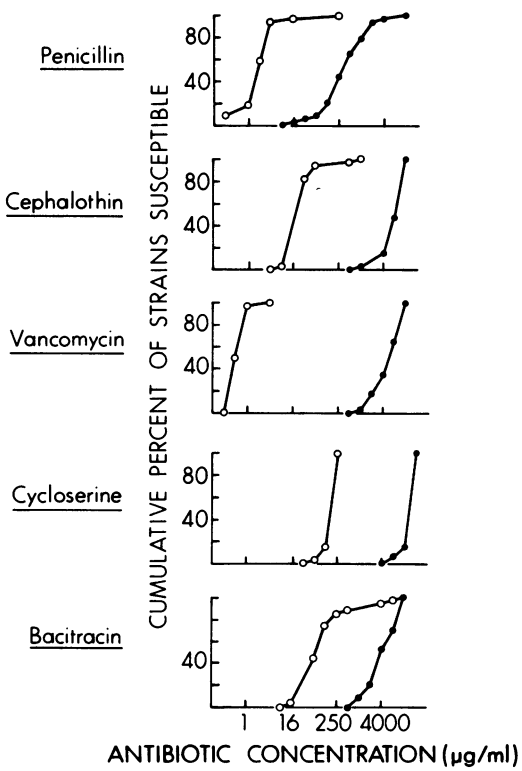

FIG. 1. *Defective killing of enterococci by five antimicrobial agents acting on the cell wall. For each drug concentration, the cumulative percentage of 34 enterococcal isolates susceptible to growth inhibition* (○) *and killing* (●) *is plotted on the ordinate.*

strated defective killing with median MBC/MIC ratios of 256 (penicillin G), 256 (cephalothin), 64 (bacitracin), ≥32 (cycloserine), and 1,024 (vancomycin).

However, the β-lactams clearly had lower MICs for the non-enterococcal group D streptococci (*S. bovis*) tested than for the enterococci (Table 2). These results contrast with those of the non-β-lactams, which had similar MICs against both enterococcal and non-enterococcal group D streptococci (Table 2).

The MICs of the β-lactams for the viridans group streptococci tested were similar to those for *S. bovis*. The MICs of the non-β-lactams were similar for both enterococcal and non-enterococcal group D streptococci (Table 2). Defective killing was not observed with the viridans group streptococci (Table 2).

DISCUSSION

Identification studies. These results establish that the organisms that we tested for antimicrobial susceptibility were enterococci, *S. bovis*, and viridans group streptococci. Although the limited sensitivity of tellurite testing for *S. faecalis* (20% false-negatives) can be explained by growth inhibition, the unexpected positive results for clot production in litmus milk by *S. faecium* (4 of 4 [Table 1]) were not associated with accelerated growth and remain unexplained. As a result, we now recommend that clot production in litmus milk should not be used to distinguish between *S. faecalis* and *S. faecium* strains.

Susceptibility studies. Resistance to penicillin killing (in the absence of β-lactamase) is a trait that distinguishes enterococci from other streptococci that commonly cause endocarditis, e.g., the viridans group streptococci (8, 16). Although several studies have documented the resistance of enterococci to killing by β-lactams (11, 23) and one previous study found enterococci resistant to killing by vancomycin (6), the mechanism of this resistance remains unknown. These studies indicate that defective killing of enterococci (an MBC/MIC ratio of ≥32) is characteristic of both β-lactams (penicillin G and cephalothin) and the group of non-β-lactams that act on the cell wall (vancomycin, cycloserine, and bacitracin). Thus, any attempt to explain the resistance of enterococci to penicillin killing must either rely upon a mechanism potentially common to β-lactams and non-β-lactams, such as the autolytic enzyme system (18), or demonstrate separate mechanisms for the two groups of drugs that both produce defective

killing (which seems considerably less likely).

These studies have also shown defective killing with *S. bovis*, a non-enterococcal group D streptococcus. Although one recent report found defective killing of two *S. bovis* isolates from patients with endocarditis (14), another study of *S. bovis* did not reveal defective killing with β-lactams (11).

In conclusion, our results are compatible with the idea that some common feature of the group D streptococci (e.g., their group-specific antigen, which is a lipoteichoic acid) (24) is responsible for their resistance to antimicrobial killing. Such an explanation would potentially relate our observations on the defective killing of enterococci and *S. bovis* to the work of Tomasz and his colleagues on the role of the autolytic enzyme system (18). Their work has shown that lipoteichoic acid inhibits the lysis of pneumococci by penicillin or cycloserine (i.e., by both β-lactams and non-β-lactams that act on the cell wall) (7, 19). However, this hypothesis does not explain the much higher MICs of the β-lactams for enterococci (17). It will also be necessary to determine the biological basis of the higher MICs required for enterococci to fully understand their resistance to penicillin. Possible explanations for the high MICs of β-lactams for enterococci include their penicillin-binding proteins (3).

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